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Extraction of zein from corn co-products

Timothy James Anderson
Iowa State University

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Extraction of zein from corn co-products

by

Timothy James Anderson

A thesis submitted to the graduate faculty

In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Buddhi Lamsal, Major Professor
Lawrence Johnson
Charles Glatz

Iowa State University

Ames, Iowa

2011

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CHAPTER 1. RESEARCH PREMISE AND THESIS ORGANIZATION

The production of grain-based ethanol has increased in recent years; the US Ethanol Industry produced 13.23 billion gallons of ethanol in 2010. In 2009, ethanol biorefineries produced approximately 30.5 million t of the co-product distillers' dried grains with solubles (DDGS). Almost all of the DDGS produced is currently utilized as low-value cattle feed for its protein and energy value. However, when recovered, corn protein in DDGS, notably the zein protein, could have industrial and high-value uses. The current commercial zein protein extraction system yields low product recovery; our objective was to improve extraction efficiency and recovery.

We began with new way to increase the extraction of zein from (DDGS). The first aim was to fractionate extracted protein, including zein from DDGS. A new solvent system utilizing a biodiesel co-product, glycerol, was designed to test its zein extractability. This solvent was a ternary solvent using either 2-propanol or ethanol with water and glycerol. Four solvents were chosen for extraction: 70% (v/v) aqueous ethanol; 55% (w/w) 2-propanol; 40% (v/v/v) aqueous ethanol; 45% glycerol, and 15% water; and 40% (v/v/v) aqueous 2-propanol, 45% glycerol, and 15% water. Both the ethanol and 2-propanol solvent systems extracted modest amounts of zein, but the solvents with added glycerol extracted much less. It was apparent that the new solvent system was not performing as well as the solvents that are currently used to extract zein from DDGS.

A new approach for extracting zein from DDGS was devised, based upon one used for extraction from corn gluten meal (CGM) (Wu et al 1997, Carter and Reck 1970). CGM is a high protein corn wet milling co-product from which zein has been extracted

commercially. Zein extraction from CGM has been plagued by low extraction efficiency of α -zein (<50%), so there is potential to increase the yield of α -zein (Wu et al 1997). We simulated the commercial extraction and chose solvents in attempt to extract higher amounts of zein. Six extraction solvents were compared for zein extraction from CGM: 88% (w/w) aqueous 2-propanol; 70% (w/w) aqueous 2-propanol; 55% (w/w) 2-propanol; 70% (w/w/w) aqueous 2-propanol, 22.5% glycerol and 7.5% water; 70% (v/v) aqueous ethanol; and 70% (v/v) aqueous ethanol with two cold precipitations at the end. A reductant treatment combined 0.5% sodium bisulfite and 0.25% NaOH was applied to further increase zein yield.

Based on zein yields from CGM, three solvents were chosen for zein extraction from DDGS. The solvents were 88% (w/w) aqueous 2-propanol, 70% (w/w) aqueous 2-propanol, and 70% (v/v) aqueous ethanol. A commercial zein extraction method from CGM and a method devised in our lab were compared for DDGS. The pretreatment of DDGS (particle size reduction and enzyme treatment) was explored. The DDGS size reduction was to determine if increased surface area of the DDGS could increase zein extraction. The enzyme treatment was a combination of cellulase and pectinase hydrolysis prior to the zein extraction. The hydrolysis was to test if polysaccharides impedes zein extraction due to a complex solute matrix and thereby reduces zein extractability from DDGS.

The thesis is presented in 3 chapters. The first chapter is a review of relevant literature on corn and corn co-products from which zein can be extracted. The review gives brief descriptions of the processing of the corn and corn co-products, zein extractions from the products, uses of zein, chemical modifications, and cross-linking of zein. The second chapter deals with the use of a new extraction method and different solvents used to extract

zein from CGM. The final chapter presents DDGS as an extraction substrate instead of CGM and uses of the best extraction solvents and the new method from the second chapter. The final chapter also explains the characterization of zein films extracted from CGM and DDGS sources.

1.1 Literature Cited

Carter, R., and Reck, D. R. 1970. Low temperature solvent extraction process for producing high purity zein. U.S. patent 3,535,305.

Wu, S. W., Myers, D. J., and Johnson, L. A. 1997. Factors affecting yield and composition of zein extracted from commercial corn gluten meal. *Cereal Chem.* 74:258-263.

CHAPTER 2. ZEIN EXTRACTION FROM CORN, CORN PRODUCTS AND CO-
PRODUCTS AND MODIFICATIONS FOR VARIOUS APPLICATIONS: A REVIEW

Modified from a paper published in *Cereal Chemistry* ¹

By

Timothy J. Anderson² and Buddhi P. Lamsal³ *

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²Graduate Student, Department of Food Science and Human Nutrition

³Assistant Professor, Department of Food Science and Human Nutrition

Iowa State University, Ames IA, 50011

* Corresponding author, Email: lamsal@iastate.edu; Ph: (515) 294-8681; FAX: (515) 294-8181.

2.1 Abstract

Corn can be fractioned to produce starch, fiber, oil, and protein in relatively pure forms. The corn kernel contains 8-12% protein, but one-half of this amount is an industrially useful protein zein. Corn gluten meal (CGM), and distiller's dried grains with solubles (DDGS) are co-products from processed corn that contain zein in various proportions and are used for zein extraction. Because zein is insoluble in water, it has found uses in many products such as coatings, plastics, textiles, and adhesives. Newer applications take advantage of zein's biological properties for supporting growing cells, delivering drugs, and producing degradable sutures and biodegradable plastics. The present paper reviews zein characteristics and nomenclature, past and current practices in processing and extraction of zein from corn products and co-products and modifications of zein for various applications.

2.2 Zein Extraction and Applications: An Overview

Maize or corn is a major cereal grain across the world; it also is the dominant crop in the United States (Anon 2010). Yellow dent has become the most utilized type of corn and varies greatly from sweet corn for human consumption. The main component of the kernel is the endosperm, which is 86.4% starch (Earle et al 1946). The starch can be extracted in relatively pure form for various food and industrial uses. Starch has mostly been used in the food sweetener market. Oils extracted from the germ can be utilized as cooking oils or in other food products. Proteins are located mainly in endosperm and germ. Different types of proteins are found in the two main constituents: albumins and globulins are centralized primarily in the germ, while prolamin-type proteins are found mostly in the endosperm.

Prolamin proteins provide nitrogen for the growing corn kernel during germination. Zein, the main prolamin in corn, was first discovered by Gorham in 1821 in *zea*, otherwise known as “Indian corn” (Gorham 1821). It was classified by Osborne (1924) as a prolamin and shown to be extractable in aqueous alcohol, such as ethanol. As production of zein was commercialized in 1939 many potential uses for zein were identified. Because of zein’s insolubility in water, resistance to grease, and glossy appearance, it was ideal for adhesives, plastics, and fiber applications. As the protein structure and properties of zein became known, zein-related research surged. However, commercial production of zein has been low with mainly two companies producing it: Freeman Industries (Tuckahoe, NY) now owned by Flo Chemical Corp. (Ashburnham, MA) and Showa Sangyo (Tokyo, Japan). Recently POET Inc. (Sioux Falls, SD) and Prairie Gold Inc. (Bloomington, IL) have introduced zein prepared by using different processes. The POET product called Inviz™ is extracted from POET's Dakota Gold® HP™ distillers’ grains, and COPE-zein from Prairie Gold Inc. is extracted from ground corn prior to the dry-grind ethanol process. Zein has normally sold for \$10-40/kg with higher purities commanding higher prices. Until new extraction methods or new products, such as the two previously listed, can prove themselves to be economically viable, zein will not likely be able to compete with synthetic plastics, which have a market price of approximately \$2/kg.

Zein is a protein that is only found in corn; however, there are proteins which share similar prolamin characteristics to that of the zein found in corn. Other cereals, such as wheat, barley, rye, and sorghum, contain prolamins with similar characteristics to zein. The extracted prolamin proteins from these cereals have each been shown to have industrial

importance, but zein is favored because of higher yields and the large volume of corn co-products available for extraction. Corn is processed using four different methods and zein extracted from these products/co-products could differ in properties and end-uses. Wet milling, dry milling, dry-grind ethanol processing, and alkaline treatment are four major corn processing methods. Corn wet milling produces a protein-rich co-product (CGM) from which zein has been extracted commercially. Dry-milled corn (DMC) separates oil and fiber-rich materials from grits. The dry-grind ethanol processing is grinding corn, subsequent saccharification, and fermentation of glucose to ethanol, leaving behind the co-product DDGS. Because of the conversion of starch to sugars and subsequently to ethanol, components, such as cellulosic materials and protein, become concentrated in DDGS. Alkaline treated corn has been mainly utilized for human consumption, and has no basis for zein extraction. Most zein extractions have been based on aqueous alcohol, but many other solvents can solubilize zein. Zein extraction schemes have been optimized for different corn products and co-products because of differences in protein concentrations and processing conditions.

Zein extractions are a complex balance of yield, quality, and purity. Yield refers to the amount of zein extracted; commercial zein until recently has been composed primarily of α -zein, and purity is the protein content in extracted zein (Pomes 1971). Commercial zein tends to be of high purity, but yields are low. The production and properties of zein have been reviewed in the past (Shukla and Cheryan 2001, and Lawton 2002). The review by Shukla and Cheryan (2001) has an overview of zein properties, extractions, and applications. The review by Lawton (2002) includes data on extractions and zein-solvent interactions, but

mainly focuses on the numerous industrial applications for zein. Since the last reviews, the zein extraction has shifted towards the dry-grind ethanol processes, which is emphasized through new commercial zein products from DDGS processes. Also the practical uses of zein demand a product low in pigment and odor. The objective of the present review is to critically evaluate the recent literature on zein extraction from corn, various corn products and co-products. We also discuss the corn zein classification and properties, treatments to increase zein extraction yields, and zein modifications for industrial applications.

2.3 Corn Proteins and Their Classifications

Corn production in the United States was approximately 335 billion kg in 2009 (Dougherty and Honig 2010). Oil, starch, fiber, and protein are the major constituents of corn, with protein at 8-12% w/w of a corn kernel (Earle 1977). Based on the production of the harvest in 2009, approximately 30.2-40.2 billion kg of corn protein was utilized for zein extraction or to supplement animal feed.

Endosperm, germ, bran, and tip cap are the four main components of the corn kernel, and each contains protein in varying amounts. The largest part of the seed is the endosperm, which constitutes 81.9% of the total mass and contains 86.4% starch and 9.4% protein (db) (Earle et al 1946). The germ comprises about 12% of the mass of the total seed and contains about 34.5% oil, 18.8% protein, 10.1% ash, 10.8% simple sugars, and 8.2% starch (db) (Earle et al 1946). The bran and tip cap are ~6% of the total mass of the seed and contain mostly fiber (db). The bran contains 7.3% starch, 3.7% protein, and 1.0% oil (db). The tip cap contains 5.3% starch, 9.1% protein, and 3.8% oil (db) (Earle et al 1946). According to Wilson (1987), the endosperm contains the prolamin zein, which accounts for 60% of the

total protein, glutelins (26% of total protein), and albumins and globulins (6% of total protein). The germ protein contains mainly albumins and globulins, each at 30%, 23% glutelins and 5% prolamins (Lasztity 1979). It should be noted that zein exists only in the endosperm; prolamins found elsewhere is either due to contamination or another prolamins protein that is not zein.

Classification of cereal grain proteins was first established by Osborne (1924), who reported four different kinds of protein based on solubility. Albumins dissolve in pure water; globulins do not dissolve in pure water, but rather in dilute salt solutions; prolamins dissolve in 70% ethanol; and glutelins are soluble in dilute acid or base. The albumins and globulins are considered biologically active proteins. They regulate and control seed metabolism. The prolamins and glutelins are major storage proteins that contain nitrogen for seed germination (Tsai et al 1980). These two categories of protein comprise 80% of the nitrogen in the corn kernel (Tsai et al 1980). Even with newer protein classification methods, these four main categories of proteins are still the basis for corn protein classification.

The solubility model Osborne (1924) developed was far from perfect. Many of the proteins dissolved in more than one solvent, some could not dissolve at all, or some proteins, such as β - and γ -zein, that dissolved in aqueous alcohols with reducing agent were classified as glutelins (Lawton and Wilson 2003) (Table 2-1). An important improvement upon Osborne's method was accomplished by Landry and Moureaux (1970). Their extraction method extracted all but 5% of the total proteins and took Osborne's extraction further by using the reducing agent 2-mercaptoethanol to help extract glutelins in an aqueous alcohol system. Later, other extraction procedures were developed to separate and understand the

Table 2-1

Corn Protein Fractionation Based on Osborne (1924) Solubility Principles

Protein Class	% of Total Protein				
	Osborne and Mendel (1914)	Mertz and Bressani (1957)	Paulis et al (1969)	Hansel et al (1973)	Wall and Paulis (1978)
Albumin	7.8	12.4	7.8	2.3	4.7
Globulin	-	-	-	2.3	3.5
Prolamins	50.0	33.9	37.6	57.5	45.8
Glutelins	38.2	36.8	43.6	31.2	38.0
Residue	4.0	16.9	11.0	5.8	9.0

Prolamin and glutelin fractions (Paulis and Wall 1971, Sodek and Wilson 1971, Paulis et al 1975, Paulis and Wall 1977). As studies showed that prolamins could be extracted by aqueous alcohol or aqueous alcohol with reducing agent, confusion in nomenclature arose because glutelins could also be extracted under the same conditions as prolamins with reducing agent (Lawton and Wilson 2003).

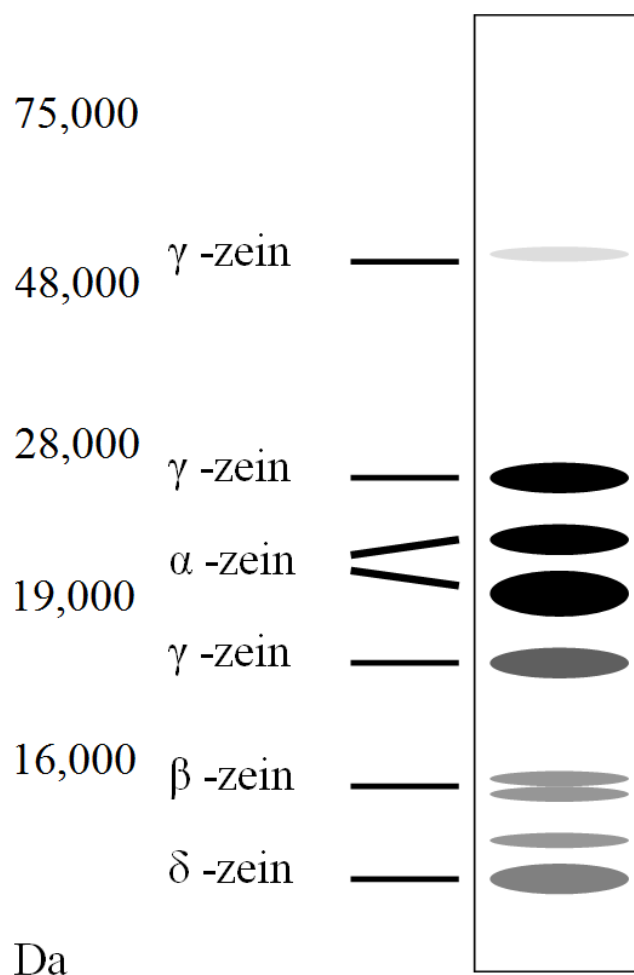
Zein is a family of many similar proteins that are most commonly classified based on solubility and identified by their molecular weights against known standards in gel electrophoresis. Zeins were thought to be prolamin proteins, but with modern classification models, zein includes both prolamin and proteins that are soluble in aqueous alcohol and a reducing agent. Because these protein fractions also are part of the zein protein body, they are included within the zein nomenclature (Wilson 1991). These classification methods were proposed by Wilson (1985) and Esen (1987).

The first method devised by Wilson (1985) used two different solubility profiles based upon zein solubility in aqueous alcohol and aqueous alcohol with 2-mercaptoethanol.

Zeins were characterized based on molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The highest molecular weight (MW) protein was A-zein (21,000-26,000 Da) followed by B-zein (18,000-24,000 Da). These two bands were both soluble in aqueous alcohol without reducing agent. The two bands soluble only in aqueous alcohol with reducing agent were C-zein (15,000-18,000 Da) and D-zein (9,000-10,000 Da). Proteins soluble in water after being reduced were identified as reduced soluble protein (RSP). RSP-1 had a molecular weight of ~27,000 Da and RSP-2 was ~58,000 Da. Later RSP-1 was renamed as E-zein (Wilson 1991).

The second method introduced by Esen (1987) classified zein based upon solubility in aqueous 2-propanol with reducing agent. Similar to Wilson's classification scheme, zeins were identified with gel electrophoresis. All zein fractions from corn endosperm dissolved in 60% 2-propanol v/v with 1% β -mercaptoethanol. α -Zein was the fraction soluble in 50-95% (v/v) 2-propanol and insoluble in 30% (v/v) 2-propanol with 30 mM sodium acetate at pH 6.0, and with MW bands within 21,000-25,000 Da and one at 10,000 Da. β -zein was soluble in 30-85% 2-propanol (v/v) with reducing agent and was insoluble in 90% 2-propanol and 30% 2-propanol with 30 mM sodium acetate at pH 6.0 and gave MW bands at 17,000-18,000 Da. γ -Zein was the fraction soluble in 0 to 80% 2-propanol with reducing agent; it was also soluble in 30% 2-propanol with 30 mM sodium acetate, unlike, α - and β -zein. The γ -zein had MW bands at 27,000 Da. Later, Esen (1990) reclassified the 10,000 Da α -zein band as δ -zein and changed the 18,000 Da β -zein band to be considered γ -zein (Fig. 2-1). The band seen in Figure 2-1 at \approx 48,000 Da, considered to be γ -zein, is a dimer of some of the smaller MW proteins.

Figure 2-1



Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of zein protein fractions from corn endosperm using extraction method of Wallace et al (1990). Adapted from Hamaker et al (1995).

There are large disparities when comparing the two nomenclature systems above.

Esen (1987) assigned α -zein as the zein that accounted for both A-zein and B-zein in Wilson's (1985) classification. The β -zein by Esen (1987) corresponded to the C-zein by Wilson (1985). Esen's (1987) γ -zein was the equivalent of Wilson's (1985) RSP-1, which was not considered a prolamin. Lastly, the δ -zein classified by Esen (1990) corresponded to the D-zein by Wilson (1985). The Esen classification has been further refined to no longer

represent only 2-propanol solubilities (Esen 1990). Zeins are now classified primarily on SDS-PAGE migration, amino acid structure, and complimentary DNA (Mohammad and Esen 1990). Of four zein fractions that were proposed by Esen (1987), α -zein is the most prevalent followed by γ -, β -, and δ -zein. These proteins make up ≈ 71 -85%, 10-20%, 1-5%, and 1-5%, respectively, of the total zein (Wilson 1991).

2.4 Zein Structure and Protein Body

α -Zein, which is the most abundant prolamin in corn, is also the most widely used. α -Zein is the only zein present in zein produced industrially (Wilson 1988). This fraction has a unique amino acid sequence and structure, which allows it to be of many industrial uses. α -Zein contains over 50% nonpolar amino acid residues and contains 9-10 tandem repeats of helical segments of these non-polar residues linked by polar turns high in glutamine (Argos et al 1982).

The proposed tertiary structure of hydrogen-bonded α -helices and the tandem repeats has been evaluated by surface adhesion with zein using hydrophobic and hydrophilic surfaces binding zein (Wang et al 2008). A recent study of the α -zein Z19 species by Momany et al (2006) proposed a 3-dimensional model in alcohol/water that showed a protein with 9 helical repeats and ~ 35 -60% helical character, and oblong structure with an aspect ratio of $\sim 6:1$. The helical repeats form a triple superhelix where lutein is within the core to stabilize the protein. Even though most characterizations of α -zeins show that it contains two electrophoresis bands considered Z19 and Z22, these α -zein bands hide a much more complex protein array which can be seen using 2-dimensional electrophoresis (Consoli and Damerval 2001).

Two-dimensional electrophoresis techniques show that there are many different types of zein, however, has difficulty in characterizing their true molecular weights due to inconsistencies with protein migration within the gel matrix. In recent years, mass spectrometry has become important in identifying protein molecular weights, including those of zein. Adams et al (2004) showed that α -zein analyzed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry found 9 different α -zein proteins ranging in true molecular weights of 23,359-27,128 Da. Five of these were considered Z19 zeins, and 4 were Z22 zeins. Data from capillary electrophoresis mass spectrometry discerned even more Z19 and Z22 α -zeins (Erny et al 2007). Eleven Z19 zeins were accounted for, along with 8 different Z22 α -zeins.

Protein bodies are the means for storing of prolamins in corn kernels. The spatial distributions of zeins has been postulated in these bodies based upon zein degradation during germination (Mohammad and Esen 1990). The use of zein-specific antibodies indicated that certain zeins degraded at different rates during germination. γ -Zein degraded the fastest placing them on the outside of the protein body. β -Zeins were the next slowest putting them in a second layer, δ -zein and α -zein both degraded slowly placing them most likely together at the core of the body. Some work used immuno-staining and microscopy methods to determine the placement of the zein proteins within the protein bodies (Lending and Larkins 1989, Thompson and Lending 1989). These studies found that protein bodies within the subaleurone layer of cells of the corn endosperm contain small protein bodies with mainly β - and γ -zeins with little or no α -zein. The protein bodies found in cells further from the

aleurone cell layers were larger and had an outer layer containing β - and γ -zeins and an inner core of α -zein.

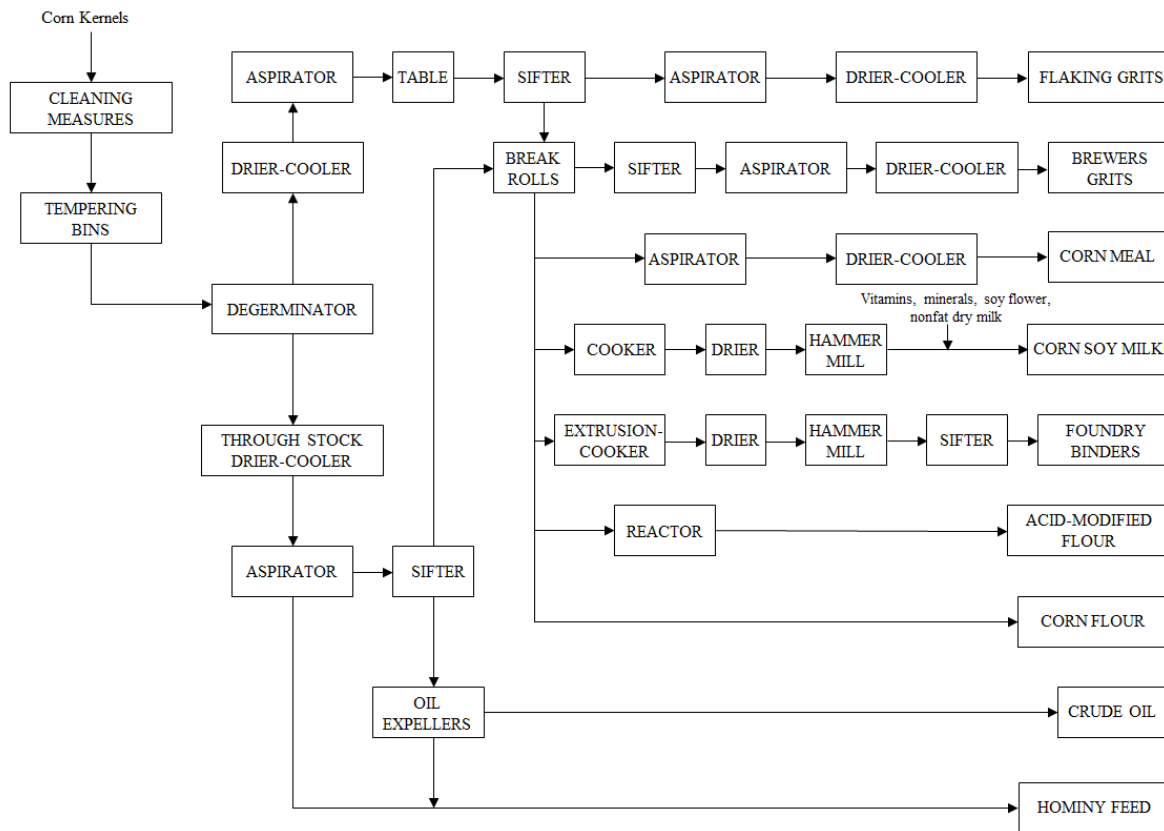
2.5 Corn Products and Co-products High in Zein and their Processing

2.5.1 Dry-milled corn

Dry-milled corn (DMC) is water-tempered corn grits where the corn endosperm has been separated from germ and pericarp through the milling process (Rausch et al 2009). It is a good material from which to extract zein because it has not been exposed to high heat, which may affect the zein protein (Rausch et al 2009). A negative aspect is that DMC contains a low amount of protein. Total protein content of DMC was shown to be 6.8-8.0% w/w of the milled corn based on hybrid used (Rausch et al 2009). The fact that DMC has low protein concentration is not always true; some varieties have much higher protein contents. Endosperm protein concentrations for two inbred corn varieties, W64A+ and W64Aae, a homozygous for the recessive gene ae, have high amounts of protein (Wolf et al 1975). The protein concentrations were approximately 13.1% in W64A+ and as high as 18.7% in W64Aae (Landry et al 2002, Wolf et al 1975).

Commercial corn dry-milling is done by three different processes; these are the full-fat milling process, bolted milling process, and tempering-degerming milling process (Duensing et al 2003). The process shown in Figure 2-2 is the tempering-degerming milling process, which can produce a wide array of products.

Figure 2-2



Flow diagram of corn tempering-degerminating process (adapted from Wells 1979)

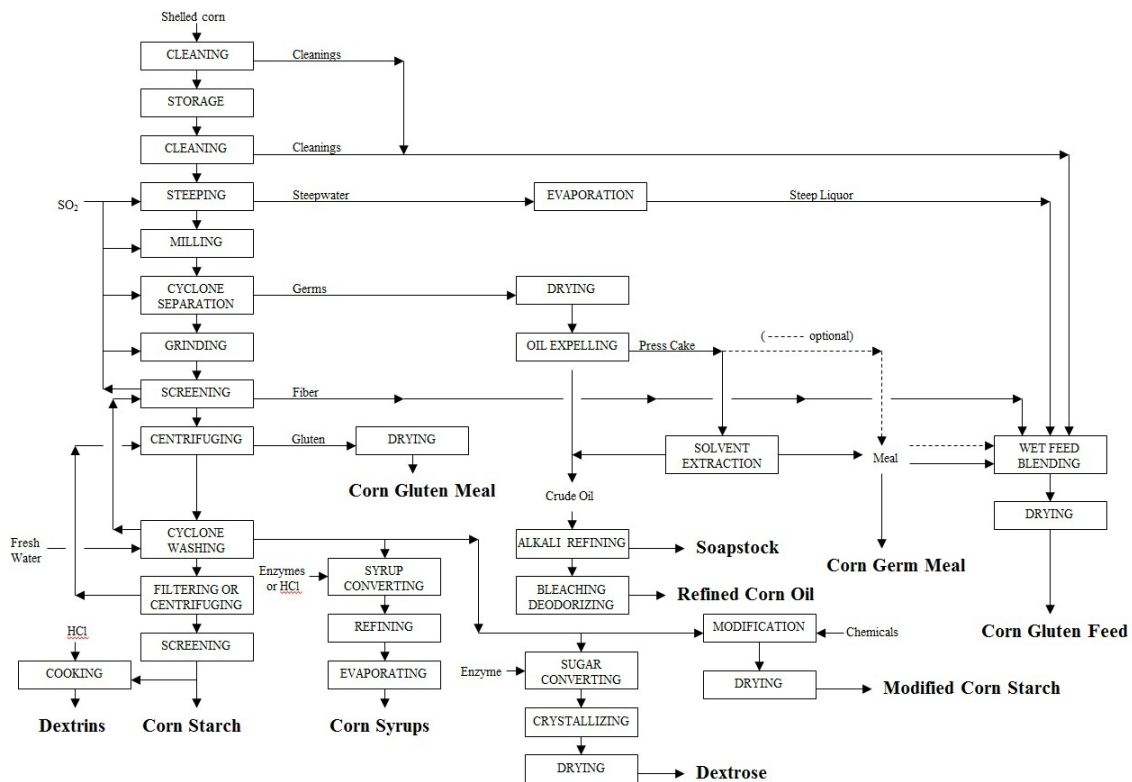
The process involves tempering corn with water to increase moisture to $\approx 22\%$; this increase in moisture aids the separation of germ from corn when using a degerminator. The corn is ground, and then a series of sieves separates grits or small pieces of corn endosperm from fiber and germ. The method separates the dry-milled corn into five different fractions: large grits, small grits, fines, germ, and pericarp. Whole dry-milled corn is the sum of all five fractions, and endosperm is the sum of the grits and fines.

Many zein extractions have used whole ground corn (Selling and Woods 2008, Shukla et al 2000). The corn is dried in a low temperature convection oven (49 °C), not high enough to alter zein physiology. The physical grinding does not harm the protein, due to the minute size (1.4 to 1.8 μm of the protein bodies (Wolf et al 1969). These protein bodies house the extractable zein in corn products (Duvick 1961). When whole DMC is extracted with 70% (v/v) ethanol without reducing agent, mainly α -zein proteins along with small amounts of β -zein are extracted because the disulfide bonds in the zein have not been broken with a reducing agent (Tsai 1980). The extract was called “native” zein and contained α -zeins along with dimers ($\approx 50,000$ Da) and trimers ($\approx 75,000$ Da). This “native” zein, however, should not be confused with zein in a truly native state, which would be packed within the protein body. There is evidence that extracting DMC without a reducing agent can extract small amounts of β -zein along with the α -zeins (Parris and Dickey 2001). This extract can be reduced to the α -zein constituents when a reducing agent is used to treat “native” zein (Tsai 1980). Reducing agents used with 55% (v/v) aqueous 2-propanol extract zein profiles that contain more zein proteins, which is called total zein (Wilson 1985). This total zein fraction extracts not only α -zein, but also β -zein, γ -zein, and δ -zeins. If extraction of only α -zein is the goal, “native” zein extractions are ideal, but yields are low. Total zein extractions yield more zein, but the other three fractions of zein are also extracted along with α -zein, reducing its purity.

2.5.2 Corn wet milling and CGM

Corn wet milling is another process, which creates a co-product that is rich in zein. The gluten meal is the component that contains the greatest content of protein along with zein. While commercial samples can contain 62 to 74% protein (Wu et al 1997b), pilot-plant scale operations can produce gluten meal that usually has 50 to 54% protein (Wu et al 1997a). The commercial wet milling process produces many high value products besides the gluten meal, which contains nearly all of the zein (Fig. 2-3).

Figure 2-3



Wet-milling process flow diagram (courtesy of L.A. Johnson, the Center for Crops Utilization Research, Iowa State University).

The process of wet milling alters zein protein in a multitude of ways that affect both extractability and properties of the protein. The steeping process helps facilitate the separation of fiber and germ. When steeping the corn, reducing agents, such as SO_2 , are used to break the disulfide linkages between proteins, which help weaken the endosperm and allow better starch separation (Cox et al 1944). Zein proteins along with others become modified by the reduction of these disulfide bonds. Drying CGM also affects zein properties; the redness in color of CGM is correlated with degree of drying. Excessive drying can reduce the yield of α -zein (Wu et al 1997b). The steeping solution does not fully penetrate the kernel so as to reduce all proteins but the cleavage of disulfide bonds affects the abilities of solvents to extract zein (Landry et al 1999). This incomplete reduction does not allow all zeins to be extracted from CGM without a reducing agent; to do so, a solvent, such as 60% (v/v) 2-propanol, and a reducing agent, such as 2-mercaptoethanol, would be necessary (Parris and Dicky 2001). Increasing the solvent concentration of 2-propanol to 90% precipitated β -zein and γ -zein along with a fraction of the α -zein. The data suggested that only high concentrations of alcohols that solvate α -zein should be used so that extraction of β -zein and γ -zein are minimized.

2.5.3 Dry-grind ethanol

The dry-grind ethanol process is utilized to produce ethanol from corn. The co-product of this process is called DDGS (Kwiatkowski et al 2006). This process is used for fuel ethanol and beverage alcohol. Cereal grains, such as corn, are as much as 60-75% starch making them ideal for fermenting and producing ethanol (Singh et al 2002). The conventional dry-grind ethanol process is a harsh process and could affect zein in a many

ways. Milled corn is combined with water, thermally-stable α -amylase, ammonia, and lime to form a slurry. The mixture is sent to a liquefaction vessel where the starch is cooked and gelatinized; the time-temperature combinations for liquefaction vary, e.g., up to 165 °C for 3-5 min for very high-temperature cooking or 90-105 °C for high-temperature cooking with holding at 90 °C for 1 to 3 h (Whitlock 2009, Robertson et al 2006). The cooked mash is then cooled to 60 °C and glucoamylase is added to produce glucose during saccharification before fermentation by yeast. After fermentation, the composition of the DDGS is different than the original corn. These DDGS constituents can vary between processing plants and also on process variations, if any, within a plant. One study of DDGS from six different dry-grind plants of both fuel and beverage ethanol variety contained 7.9-15.1% oil, 28-30% protein, 38-49% neutral detergent fiber, 14-19% acid detergent fiber, 3.7-4.6% and ash (Singh et al 2002). During the dry-grind ethanol process the corn components change considerably from ground corn to DDGS (Han and Liu 2010) (Table 2-2). Han (2010) found that yeast protein contributed approximately 20% to the protein in DDGS.

Modifications have been proposed to the dry-grind ethanol process by Singh et al (2005). The modifications were to remove intact germ and fiber prior to the dry-grind process to obtain high quality co-products. The germ and fiber contribute little to the fermentation process because starch is the main corn component utilized to produce ethanol. The modifications by Singh et al (1999, 2005) were termed “quick germ” and “quick fiber” processes. After germ and fiber removal the dry-grind ethanol process was performed the same as the conventional method.

Table 2-2

Amount of Protein in Step-Wise Processes of Dry-Grind Ethanol Plants^a

Ethanol Processing Streams	Protein Content (%)		
	Ethanol Plant 1	Ethanol Plant 2	Ethanol Plant 3
Ground corn	7.7	7.8	7.5
Cooked slurry	9.3	8.9	8.1
Liquefied mass	9.8	9	8.1
Saccharified mass	12.1	9	NA
Fermented mass	29.4	26.6	27.2
Whole stillage	29.5	26.8	26.6
Thin stillage	22.9	17.2	21.1
Distiller solubles	21.3	17	21.2
Distiller grains	33.4	32	30.2
WDGS ^b	27.7	NA	26.9
DDGS	29.5	29.4	26.7

^a Protein contents of corn samples, intermediate products, and ethanol co-products from processing streams produced in three dry-grind ethanol plants. Data adapted from Han and Liu (2010).

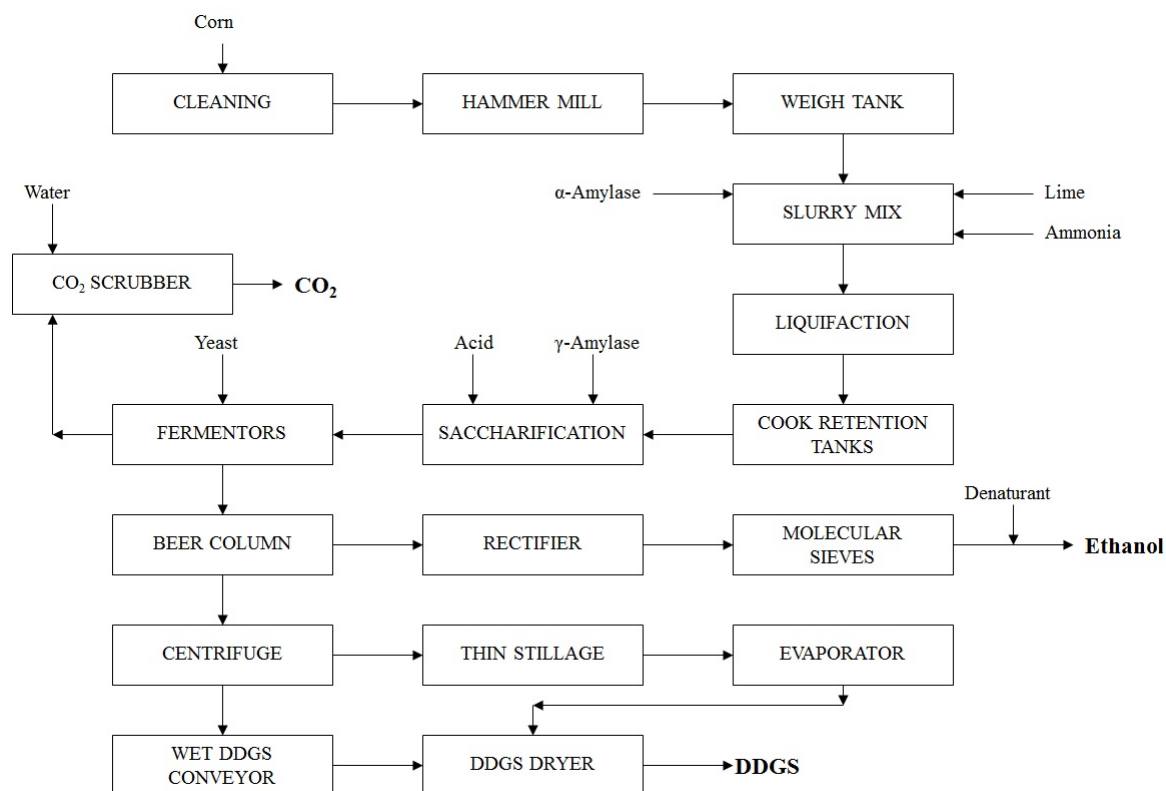
^b Wet distiller's grains with solubles (WDGS)

The E-mill process went further than the “quick germ” and “quick fiber” processes. Enzymes, such as amylase and protease, were added to the ground corn and water and incubated. Germ and pericarp fiber were removed, and then the slurry was sieved to collect

endosperm fiber prior to the rest of the process, which was conventional. The protein concentrations in the recovered DDGS were 28, 36, 49, and 58% for the conventional, “quick germ” and “quick fiber”, and E-mill processes, respectively (Singh et al 2005). This E-mill DDGS has protein content nearly that of CGM and much greater than DDGS from the conventional dry-grind process (Kim et al 2008). There is no published data for extraction of zein from DDGS produced through the “quick germ”, “quick fiber”, or E-mill processes; however, the quality of the zein extracted from DDGS of E-mill processes could be inferior because of hydrolysis or modification to zein from the proteases used to separate protein bodies from starch. It will not be clear whether zein from this DDGS can be used industrially until the proteins are characterized. If the zein could be used industrially, it may contain unique properties due to the fact that it has not undergone a sulfite steep as has zein obtained from CGM. Breaking of disulfide bonds in zein may change the conformation of solvated zeins in solution by opening up their native conformation and thus alter the properties of the extracted zein. If the disulfide bonds are not broken, less concentrated solvents, such as 70% (v/v) ethanol, can extract α -zein without extracting γ -zeins (Tsai 1980).

The dry-grind ethanol process involves drying DDGS at high temperatures, which could affect the zein extractability. A study by Kwiatkowski (2006) detailed a conventional dry-grind ethanol process (Fig. 2-4). When the temperatures are around 90 °C, the shape of the protein bodies are not altered, it would take mechanical means, such as extrusion or pressing, to cause leakage of α -zein from within the protein body or merging of protein bodies (Batterman-Azcona and Hamaker 1998). Cooking up to 70 °C did not greatly affect protein extractability, but when cooking at 100 °C, the extraction yield of protein decreased

Figure 2-4



Dry-grind ethanol flow diagram (adapted from Kwiatkowski 2006)

(Batterman-Azcona and Hamaker 1998). The fermentation with yeast is also another potential setback for degradation or alteration of zein in the process. The fermentation yeast converts starches to ethanol, but yeast need nitrogen for vigorous growth; urea or ammonia have been added as supplements (Jiranek et al 1995).

Proteases may be added to the fermentation to breakdown corn protein to aid yeast uptake of nitrogen considering their lack of producing their own proteases (Bothast and Schlicher 2005). This proteolytic activity may hydrolyze the zein protein. Papain can hydrolyze zein into low-molecular-weight peptides with molecular masses less than 10,000

Da (Saito et al 1988). The rest of the brew after fermentation/distillation is separated into thin stillage and distiller's grains. The thin stillage is evaporated and combined with distillers' grains before drying to become DDGS. The drying of distiller's grains can further degrade zein because of a harsh drying temperature, which quickly reduces the moisture from 65 to 10-12% (Bothast and Schlicher 2005) and the high heat conditions can induce cross-linking of zein protein in the protein body. With all the potential for changes to zein in DDGS, it is the least preferred source to extract zein among the three methods previously described. Nevertheless, zein proteins have been extracted at lab/bench-scales from DDGS and characterized via SDS-PAGE (Wolf and Lawton 1997, Xu et al 2007).

2.6 Extractions of Zein from Corn Products and Co-products

2.6.1 Zein Extraction Solvents

Many different solvents can be used to extract zein. Much of the zein solvent solubility was determined based on the solubility of commercial zein. There are three different types of solvents for extracting zein: primary solvents, secondary solvents, and ternary solvents (Evans and Manley 1941, Manley and Evans 1943, Evans and Manley 1944) (Table 2-3). A primary solvent is a compound which could dissolve zein alone in a concentration > 10% (Evans and Manley 1941). To stand alone as a solvent for zein, it needs to be able to interact with the amino acids of zein so that it could simultaneously dissolve both polar and non-polar amino acids in zein. Secondary solvents are organic compounds classified into two different classes; one group must be added to water and the other added to a lower aliphatic alcohol to gain solvation power (Manley and Evans 1943). These solvents

rely on the organic compound to provide interaction with the non-polar amino acids and water to interact with the polar amino acids.

Table 2-3

Categories of Solvents that Extract Zein

Solvents for Zein	Class A- Primary Solvent^a	Class B- Secondary Solvent^b	Class C- Secondary Solvent^c	Class D- Ternary Solvent^d	Class E- Ternary Solvent^e
Acetaldehyde			•	•	
Acetamide	•				
Acetic acid	•				
Acetone		•	•	•	
Acetonyl acetone		•		•	
2-amino-2-ethyl-1,3-propandiol	•				
2-amino-2-methyl-1-propanol	•				
Aniline	•				
Benzene			•	•	
Benzyl alcohol	•				
Benzyl Cellosolve	•				
1,3-Butanediol					•
1,4-Butanediol					•
2,3-Butanediol					•
<i>n</i> -Butanol		•			
<i>t</i> -Butanol		•			

Table 2-3 (continued)

Solvents for Zein	Class A- Primary Solvent ^a	Class B- Secondary Solvent ^b	Class C- Secondary Solvent ^c	Class D- Ternary Solvent ^d	Class E- Ternary Solvent ^e
<i>s</i> -Butanol		•			
Butylamine	•				
Butyraldehyde				•	
Ethylene glycol	•		•		•
Ethylene glycol monoethyl ether	•			•	
Ethylene glycol monomethyl ether	•				
Ethylenediamine	•				
Formaldehyde				•	
Formic acid	•				
Furfuryl			•		
Furfuryl alcohol	•				
Glycerol	•				
Glycerol furfuryl	•				
Glycerol- α - γ -dimethyl ether	•				
Glycerol- α -monochlorohydrine	•				
Glycerol- α -methyl ether	•				
Glycerol- α -phenyl ether	•				
Hexylene glycol					•
β -Hydroxyethylaniline	•				
Hydroxyethylethylenediamine	•				

Table 2-3 (continued)

Solvents for Zein	Class A- Primary Solvent^a	Class B- Secondary Solvent^b	Class C- Secondary Solvent^c	Class D- Ternary Solvent^d	Class E- Ternary Solvent^e
2-Hydroxymethyl-1,3-dioxolane	•				
Isobutanol		•			
Isopropanol		•			
Lactic acid	•				
Methanol	•	•			
Methyl acetate				•	
Methyl ethyl ketone			•		
Methyl lactate	•				
Methylene chloride			•		
Monoethanolamine	•				
Monoisopropanolamine	•				
Morpholine	•				
Morpholine ethanol	•				
Nitroethane			•	•	
Nitromethane			•	•	
Phenol	•				
Phenylethanolamine	•				
<i>n</i> -Propanol		•			
Propionic acid	•				
Propylene chlorohydrin	•				
Propylenediamine	•				

Table 2-3 (continued)

Solvents for Zein	Class A- Primary Solvent ^a	Class B- Secondary Solvent ^b	Class C- Secondary Solvent ^c	Class D- Ternary Solvent ^d	Class E- Ternary Solvent ^e
Propylene glycol	•		•		•
Pyridine	•				
Resorcinol monoacetate	•				
1,1,2,2-Tetrachloroethane			•		
Triethylenetetramine	•				
Tetrahydrofurfuryl alcohol	•				
Toluene			•		
Triethylene glycol	•				
Triisopropanolamine	•				

^a Primary solvent: compound that alone dissolves zein in a concentration higher than 10%

^b Secondary solvent: dissolved zein when solvent was combined with water

^c Secondary solvent: dissolved zein when solvent was combined with a lower aliphatic alcohol

^d Ternary solvent: dissolved zein when solvent was combined with water and a lower aliphatic alcohol

^e Ternary solvent: dissolved zein when E-class solvent was combined with water, and another class-E solvent

Compiled data of CPSC (1949), Evans and Manley (1941), Evans and Manley (1943), Evans and Manley (1944)

The ternary solvents are similar to the secondary solvent's two classes of compounds. The ternary solvents must be a combination of solvent, water, and lower aliphatic alcohol.

A comprehensive list of primary, secondary, and ternary solvents can be found in the review by Lawton (2002). Most commonly, binary solvents of alcohols, such as ethanol and

2-propanol, are used for extracting of zeins. These two solvents are easy to separate from zein to aid in an easy recovery (Swallen 1941). When evaluating a solvent or solvents, it is important to understand recovery and recyclability of solvents. If the final material is a zein concentrate, solvent cost would be very important because of solvent being lost with the product. Mckinnery (1958) noted that α -zein is soluble in 95% (v/v) ethanol and 85% (w/w) 2-propanol. α -Zein, β -zein, γ -zein, and potentially δ -zein were soluble in 60% (v/v) ethanol. This combination of zein proteins in solution caused gelation (Pomes 1971).

2.6.2 Zein Extractions from DMC

Most of the zein extractions have been based on dry-milled corn. The first zein extractions were from dry ground corn as described in a patent by Osborne (1891). In laboratory-scale operations, zein could be dissolved in 80-85% ethanol and concentrated by evaporating the alcohol. The zein then was redissolved in 90% ethanol and reduced, and added to absolute ethanol to remove the pigment and lipid. Osborne (1891) noted that the extraction was not commercially practical due to only 6-7% zein yield of the total corn meal. His method of extraction was similar to the laboratory-scale method of the time, but used gluten meal that contained higher protein content. He extracted the zein by means of 95% (v/v) ethanol and recovered the dissolved zein by pressing the spent gluten meal, and precipitated the zein from the solvent by adding it to water. This method extracted mainly α -zein and lipid impurities were precipitated out by cooling.

A more recent extraction done by Shukla et al (2000) using ethanol as solvent identified many parameters for optimal zein extraction from milled corn prior to dry-grind ethanol processing. The material used was whole ground corn obtained from a dry-mill

facility. Processes optimized were based on extraction time, temperature, ethanol concentration (% v/v), solvent:solids ratio, and number of extractions. They utilized a quadratic mathematical model to identify optimal temperature (45.6 °C), ethanol concentrations (68.1%), solvent:solids ratio (7.8 ml/g), and time for extractions (54.8 min). The yield of zein was 60% of the total zein with 50% protein purity. The quality of the zein extracted, however, was questionable because a) the extraction solvent potentially extracted small amounts of β -zein and b) the prepared SDS-PAGE gel was indiscernible to allow for meaningful characterization of proteins extracted. Because the corn was not steeped, the β -zein may not be detrimental to the final zein quality, but there was no mention of zein solubility or functional properties. The protein purity of the extracted zein was low at 50%, lipids were co-extracted with zein, the purity of the protein would likely have been improved if the corn had been defatted prior to the extraction.

An extraction of DMC by Parris and Dickey (2001) used various pre-treatments of substrate to explore differences in zein extraction and analyzed film qualities of the extracted zein. The pretreatments were 0.5% sulfuric acid, 0.55% lactic acid plus 0.2% sulfur dioxide, 0.5% sodium bisulfite, and 0.5% sodium sulfite at 50 °C in water for 6 h. Zein yields from the pretreated DMC using 70% ethanol at 60 °C for 2 h were 1.8, 2.7, 1.3, and 2.1% for 0.5% sulfuric acid, 0.55% lactic acid plus 0.2% sulfur dioxide, 0.5% sodium bisulfite, and 0.5% sodium sulfite treatments, respectively. The isolate from these zein extractions were 80-85% protein, 15-20% lipid, and < 0.25% starch. The authors also studied the solubility of zein extracted from DMC using 70% (v/v) ethanol under extraction temperatures of 23 and 60 °C along with NaOH. They found that zein solubilities were 64 and 76% when extracted at 60

°C with and without NaOH, respectively, while at 23°C the zein solubilities were 96 and 95% with and without NaOH, respectively. A gradient was set up to study the SDS-PAGE protein profile of zein from DMC using extraction solvents that were 95, 90, 80, 70, 60, and 50% (v/v) aqueous ethanol. There was a gradual increase in presence of β -zein in the samples as the proportion of alcohol in the solvent dropped. These solubility characteristics showed that extraction procedures, such as by Shukla et al (2000) with 70% (v/v) aqueous ethanol from dry milled corn, may leave as much as 24% of the recovered zein rendered insoluble. Absent from characterization were SDS-PAGE profiles of zein extracted from CGM at solvent concentrations of 50, 60, 70, 80, 90, and 95% (v/v) ethanol concentrations similar to that for ground corn.

Extraction of fat from milled corn is important for producing a useful oil stream and increasing a purity of extracted corn protein. Early work exploring the solubility of vegetable oils in anhydrous and azeotropic ethanol and 2-propanol was done by Harris et al (1947, 1949), Beckel et al (1948), Rao et al (1955), and Rao and Arnold (1956ab). A patent by Chen and Hoff (1987) used 90-100% aqueous ethanol to remove oil from cracked corn and subsequently extract protein from the remaining residue. They used 50-70% ethanol with 0.05 - 0.15 N NaOH at 50-70 °C. If only zein proteins are to be extracted, NaOH should not be used. Zein extraction was carried out at 50 °C and 40% of the total corn protein was extracted with a solution concentration of 2.8% w/v zein. A second extraction using 50% ethanol and 0.08 N NaOH extracted an additional 20% of corn protein. This procedure was successful in extracting both oil and protein using a single solvent. Higher titers of ethanol were able to extract oil while not extracting zein. A drawback to this

procedure is that it extracted protein with an ethanol solution that may not be optimal for zein extraction. Zein in DMC extracted well with 70% ethanol, but at 50% ethanol α -zein along with its dimers and trimers are not highly soluble (Parris and Dickey 2001). Treating with NaOH may help facilitate the protein extractions, but with a potential to co-extract non-zein proteins.

Another similar procedure by Hojilla-Evangelista et al (1992a, 1992b) used a sequential oil extraction process that simulated counter current oil extraction. The process extracted > 90% of the oil, which was superior to the estimated 72% oil extracted from conventional hexane prepress extraction. Non-oil materials were co-extracted with the oil. These materials were 25-30% protein and accounted for about 10% of the protein initially in the corn. A 50% ethanol and 0.08 M NaOH solvent extracted 57% of the total protein (Hojilla-Evangelista et al 1992b, Meyers et al 1994).

Zein extraction from defatted ground corn was reported by Hojilla-Evangelista and Johnson (2003). Method A used a 4:1 70% aqueous ethanol to ground corn ratio at 60 °C for 1 h, liquid solvent was collected and cold precipitated at -18 °C, redissolved and subsequently precipitated at -18 °C once more and dried. In Method B, 4:1 70% aqueous ethanol was added to ground corn at 60 °C for 1.5 h. The supernatant was collected and concentrated through a 10,000-Da regenerated cellulose membrane by ultrafiltration, solids were air dried, then further dried in vacuum oven. When comparing the two extraction methods; the Method A yielded 24% of the extractable zein while B had a 70% yield. They mentioned that when utilizing 80% ethanol in both Methods, there was a decrease in yield down to 14% for Method A, and 44% for Method B, indicating a decrease of zein solubility

with increasing ethanol concentration. The extracted low-molecular-weight proteins, based on the SDS-PAGE analysis, however, were different even though zein was extracted using both Methods A and B using the same extracting solvent. Method A had two bands at 14,200-18,400 Da and Method B showed a band between the α -zein and 18,400 Da marker. The two bands at 14,200-18,400 Da from Method A were considered to be non-zein protein, which showed that the chilled extract contained more impurities. The comparison between these two methods seems flawed in that extraction times were different by one-half hour along with a 7,000 x g variation in centrifugation. It would seem that a true comparison would synchronize the time of the extraction of the two methods employed. They do show some merit though with Method B obtaining a higher yield of extractable zein. Method A employed cold precipitation which caused the zein to leave solution, the protein precipitated on the walls of the vessel or in solution. But the zein also could stay dissolved into the solution, or not completely precipitate out of solution. With such a low amount of zein extracted from ground corn, it may be more prudent to follow Method B and use ultrafiltration to recover zein.

2.6.3 Zein Extractions from CGM

Commercial extractions of zein have classically utilized CGM because protein contents are 61.5-74% (db) with 60-71% zein proteins (Wu et al 1997b). One of the first commercial zein extractions from CGM was described in a patent by Swallen and Haute (1938). A well-defined extraction process was later patented by Swallen (1942) detailing extraction from CGM with 85% 2-propanol in a solute-to-solvent ratio of 1:3.5 at 60 °C. The extracted zein solution and gluten were separated and the zein solution cooled to 15 °C and

filtered. The solution had much of the yellow pigment extracted in a mixture with 80 parts of hexane to 100 parts of zein solution. The hexane could be removed and the zein precipitated in water. The precipitated zein was then placed into ring dryers. The yield of zein from this method was 50% of the protein in the CGM, which is considered very high. The higher yield of zein was probably due to the use of a countercurrent extraction method, rather than batch extraction. Even after hexane extraction, the zein was not completely decolorized, but still was pale yellow due to residual pigment. The one major problem with this method was that it employed two extraction solvents, which must be separated and recycled to be profitable. This incurs large costs and the hexane/2-propanol separation to decolorize the zein can carry some zein out of the 2-propanol layer, decreasing yield. Another issue with the method is that based on the current measurements of α -zein extractability from commercial CGM using a similar solvent and method, yields are only 21-32% (Wu et al 1997b) in comparison to the 50% reported by Swallen (1942). The composition of protein describing total zein in the CGM was not detailed so this discrepancy in yield was unexplained.

Carter and Reck (1970) proposed an extraction process that is considered to be the most common commercial method based on Swallen's work (1938, 1942). Carter and Reck's method extracted zein from CGM, using 88% (w/w) aqueous 2-propanol with 0.25% NaOH for 1 h at 1:4 solute to solvent ratio and 55-65 °C. The resulting zein solution was separated from the spent CGM and subsequently chilled to -15 °C. The zein precipitated into a taffy-like solid and the supernatant was discarded. One-half of the solids was used to produce a low quality zein when the solids were dried at 0.06 atm and 50 °C. Redissolving the second one-half of the zein solids from the first precipitation in 88% aqueous 2-propanol

and performing a second precipitation produced high quality zein when dried. The yield of the total zein from both parts was about 22% of the CGM. This method has problems mostly with the means of extraction and the cold precipitation. Extractions using higher titer alcohols, such as 95% (v/v) ethanol, are good at extracting just α -zein, but the yields are low. 2-Propanol used at 85% (v/v) has a solvation potential for zein similar to 92% (v/v) ethanol (Swallen 1942). Without extracting the outer layers of the protein body, which are not soluble in those extraction solvents, the amount of α -zein extracted from the core of the protein body may be low. With commercial CGM containing 36-47% α -zein, the extraction is not very efficient, but still obtains a lot of zein because of the high amount of protein (Wu et al 1997b). A cold precipitation step works better for extraction from CGM than from DMC, but still has potential for not precipitating all zein from solution. This precipitation may further decrease the zein yield coupled with poor solvent. Even with the low extraction efficiency, this zein has good solubility characteristics and is of good quality for commercial use.

The zein produced by Carter and Reck (1970) procedure had a yellow hue; Cook et al (1996) invented a process that removed pigments. Their extraction destarched the CGM first and then washed the CGM several times with absolute ethanol to remove pigment and oil. The CGM was then washed with water and then extracted with 80% ethanol. The extract was treated with activated carbon to remove flavors and pigments. Water precipitated the zein, which was dried to be used for pharmaceutical purposes. Cook et al (1993) mentioned that after extracting the CGM with 70-90% ethanol (v/v), the extraction cake can be purified and that a glutelin byproduct can be collected. The glutelin byproduct can be used to make

products such as vegetable protein supplements. This method can extract zein without pigment; however, copious amounts of ethanol must be used to wash the pigments from the CGM and the multiple purifications. The pellet was resuspended as many as 5 times in 2 volumes of 100% ethanol to remove all of the pigment. In addition to the large amount of solvent, about 2% of zein was lost in the pigment extraction. The solvent used may perhaps affect the quality of zein extracted. Zein extracted with high titer alcohol concentrations such as 90-95% ethanol, produced high quality zein (Swallen 1942). Solvents with less ethanol may extract the other zein fractions which impair resolubility of extracted zein with the benefit of increased yield.

Another method for extracting and decolorizing zein was patented by Takahashi and Yanai (1994) of Showa Sangyo Co., Japan. The method extracted zein from CGM using 70% (v/v) aqueous acetone at 40 °C for 4 h at a solute-to-solvent ratio of 1:5. The solution was separated from the solids and concentrated by evaporation. Absolute acetone was added to the precipitate to form a honey-like consistency. The syrup was added drop-wise into an absolute acetone solution to precipitate the zein. The method recovered 20.4% yield of white zein. This method performed better than that of Carter and Reck (1970) in that the zein was mostly depigmented. Using size-exclusion chromatography of Showa zein and analyzing the eluted solvent with absorbance spectroscopy, small peaks matched those of xanthophylls (Kale et al 2007, Cheryan et al 2007). Zein may have appeared depigmented in its dry form, but when observed in solution, it still contained pigments (Sessa et al 2003). The zein obtained was stated by the authors to be of high purity, but protein purity was not divulged; also the solubility of the zein was uncertain. Evans and Foster (1945) had extracted zein

using similar aqueous acetone solutions and found that the zein had different solubility than alcohol-extracted zein. There was no evidence that the zein was actually low in solubility, but only that it may have a different solubility profile because of the difference in extracting solvents.

While most extractions of zein are done with aqueous alcohols, a method by Selling and Woods (2008) showed that glacial acetic acid could be used to extract large quantities of zein from CGM, and to a lower degree zein from ground corn and DDG. The method extracted zein from the materials using 25 g of dry solids in 75 g of acetic acid. The extraction was carried out at 25 °C for 1 h, and the supernatant was separated from the solids by centrifugation. The zein yields were 37.2, 1.2, and 3.2%, for CGM, ground corn, and DDG, respectively. The protein content of the zein from CGM, ground corn and DDG was 84, 67, and 20%, respectively. These higher yields were due mainly to lipids and pigments extracted with the zein. They concluded that acetic-acid-extracted zein had similar SDS-PAGE profile compared to zein extracted using other solvents and to commercial zein. This method seems to get large yields of what appears to be zein based on the SDS-PAGE, which showed protein bands from the acetic acid extraction to be nearly homologous to commercial zein, and zein extracted with 80% ethanol. They also demonstrated that the films of acetic-acid-extracted zein had 18 MPa tensile strengths, 11% elongation, and 293 MPa Young's Moduli. The film prepared from commercial zein had 43 Mpa tensile strength, 13% elongation, and 777 MPa Young's Modulus. This showed that the films prepared from acetic acid zein extract had physical strength properties lacking in comparison to commercial zein. Also, solubility characteristics were only demonstrated for acetic acid and no other solvent,

which does not make it clear whether the zein would be soluble in aqueous alcohols or show a similar profile for solubility. Other deleterious properties of using this solvent are that zein binds the acetic acid and may hold a pungent acid odor after a potentially expensive solvent removal.

2.6.4 Zein Extractions from DDGS

In recent years as ethanol dry-grind processes have become widely utilized, DDGS has become much more available. Wu et al (1981) gave insight into the compositions of the protein fractions of DDGS and materials at the base of still after corn ethanol distillation. Four consistent extraction methods followed with two different methods of extraction using reducing agent were employed and compared to determine protein solubility based on Landry and Moureaux (1970). Four pre-extraction procedures were carried out prior to the two extraction methods, the pre-extraction procedures consisted of a water extraction, a sodium chloride extraction, a 70% ethanol extraction, a 70% ethanol plus dithiothreitol (DTT) extraction. These four extractions used for both methods extracted 14% of the total protein. The first reducing method utilized borate, SDS, and DTT extracted only 30% of the DDGS protein while 51% of the total protein was left in the residue. The second method using NaOH and DTT at pH 11.9 extracted 28% of the protein; the next step using NaOH with SDS and DTT extracted an additional 26% protein and 18% was left in the residue. The low protein yield in comparison to corn was attributed to denaturation of the protein during alcohol distillation.

Further work on zein extractions from DDGS with reducing agents was done by Wolf and Lawton (1997). Nine different materials were extracted and compared in this work;

among them were corn flour, CGM collected in a centrifuge and air dried, CGM commercially dried in a drum dryer, whole stillage from the dry-grind ethanol process, a DDGS sample of each dry and wet material, all wet samples of whole stillage that had been freeze-dried, and three other DDGS materials that were commercially dried. The yields of crude zein from the extractions were 3.2-6.6%, but protein contents of these yields were only 37-57% and lipids and pigments co-extracted decreased protein content. The SDS-PAGE showed faint bands of α -zein in DDGS extract. The authors concluded that because of the low protein purities of the samples, integration of the zein from DDGS in biodegradable applications was not here yet. This extraction showed that extracting zein from DDGS was possible, although the yields were low with a reducing agent. Defatting DDGS would most likely be a remedy for the low purity of the extracted zein proteins.

Extraction of zein from ethanol-defatted DDGS under acidic and basic conditions with reducing agent was done by Xu et al (2007). Zein was extracted from DDGS using 70% ethanol and 0.25% sodium sulfite with the pH altered by using HCl or NaOH. The optimum yield of zein solid obtained was about 90% protein and 44% recovery of the protein in DDGS was attained at a pH of 2. The high purity of protein was promising; the method also extracted higher yield. The quality of zein thus extracted, however, is of concern with potential non- α -zein proteins being extracted. The use of a reducing agent may also liberate glutelin proteins. The SDS-PAGE of the protein in DDGS extracted at pH 2 was shown to have a similar protein profile to that of commercial zein and contained a large degree of α -zein.

One successful extraction of an utilizable zein from a dry grind method has been from DDG not DDGS. POET Inc. (Sioux Falls, SD) has been able to produce an edible zein product called InvizTM extracted from POET's Dakota Gold® HPTM distillers' grains using the BFRACTM dry-mill ethanol process (POET 2010). The resultant DDG from the method contained $\approx 40\%$ protein. The zein had many different properties from that of conventional commercial zein extracted from CGM in that it contained not only α -zein, but also β and γ -zein (POET 2010). The zein contained these fractions because it had not been steeped giving zein properties similar to that of zein extracted from DMC, which can be directly used to make films (Boundy et al 1967). The steeping process reduces intermolecular disulfide bonds, which can cause β - and γ -zein proteins containing many cysteine residues to unfold and change conformation when their thiol groups are reoxidized. The InvizTM zein is described as being slower at dissolving in aqueous alcohols than commercial zeins containing just α -zein (POET 2010). No information is available as to whether a reducing agent was used to facilitate more complete extraction of zein from the DDG.

Another recent release of a commercial zein implemented within the dry-grind ethanol process has been made by Prairie Gold Inc. (Bloomington, IL). Their product is called corn oil and protein extracted (COPE) zein or COPE-zein and is extracted from the ground corn at the frontend of the dry-grind ethanol process whereas the POET process is a backend extraction process (Cheryan 2009). The COPE process simultaneously obtains both high quality corn oil with beneficial nutrients and commercial quality zein. This extraction from ground corn uses 90-100% aqueous ethanol that extracts mainly corn oil and small amounts of zein. A second extraction of the ground corn with 60-90% aqueous ethanol

yields a majority of the zein from the corn. Ultrafiltration and nanofiltration of the extracts allows oil and zein to be collected and solvent to be recycled into the system (Cheryan 2002). Size-exclusion chromatography can be used with this technology to further purify and separate zein from the pigments and oils (Cheryan et al 2007). The stated benefit of this zein product is that it has not been altered by either steeping or fermentation (Cheryan 2009).

2.7 Zein Purification

Zein extracted conventionally with aqueous alcohols or aqueous acetone contains carotenoids including β -carotene, zeaxanthin, and lutein that give zein its yellow color (Quackenbush et al 1961, Blessin 1962, Kurilich and Juvik 1999). Decolorized zein commands higher prices and has more uses than conventional yellow zein (Sessa et al 2003). Sessa et al (2003) investigated the ability of conventional procedures to decolor zein, such as partitioning and activated carbon (Mason and Palmer 1934, Swallen and Haute 1938, Pearce 1941, Starling et al 1951). They compared the conventional methods to the newer processes of column chromatography, supercritical fluid extraction with CO_2 (SFE- CO_2), ultrafiltration/diafiltration, and subcritical propane extraction. The combination of sephadex LH-60 in column chromatography and ultrafiltration/diafiltration removed pigments effectively. The best method was to use activated carbon, but because the zein in solution was dilute, other procedures, such as SFE- CO_2 , and column chromatography also removed nearly the same amount of pigment. Recent work by Sessa (2008) showed that zein could also be deodorized as well as decolorized with activated carbon at various temperatures and that the odor component was diferuloylputrescine. When zein in solution was heated to 55 °C, color/odor compounds that bound to the activated carbon were significantly increased.

They attributed this increase to denaturation of the α -helical nature of zein, which houses lutein (Momany et al 2006). The removal of odor improves zein's marketability for applications such as gum (Sessa and Palmquist 2008). Besides activated carbon, Sessa and Palmquist (2009) used zeolites to bind color/odor components. The activated carbons and zeolites both adsorbed protein as well as the color/odor components, thus reducing the efficiency of the zein purification.

Other zein decolorizing strategies using column chromatography was to obtain high-value pure xanthophyll stream from zein prior to dry-grind ethanol processing (Cheryan 2001, 2002). Cheryan's method to purify xanthophylls used extensive ultra/nanofiltration and diafiltration to obtain zein at > 90% purity. To simplify xanthophyll extraction, Kale et al (2007) and Kale and Cheryan (2009) used LH-20 resin in a column. They found that zeins eluted first, non-zein impurities second, and xanthophylls last with good resolution. Most membrane separation methods, such as ultra/nanofiltration, gave lower zein purity and yield, however, with size-exclusion chromatography both higher yield and purity > 90% were possible.

2.8 Zein Modifications

2.8.1 Zein Plasticization

Zein protein without a plasticizer produces brittle solids; thus it must be plasticized to provide flexibility. The α -zein protein contains a majority of non-polar residues, up to 53.2%, but many other residues are polar, the most prevalent being glutamine (Geraghty et al 1981). An understanding of the amino acids present and their relative polarities determine

what compounds can be used to plasticize zein. Parris and Coffin (1997) showed that a combination of glycerol and poly(propylene glycol) (PPG) increased zein film flexibility. They compared the films' water vapor permeability, low water vapor permeability values are ideal for packaging applications. The values of water vapor permeability for 15% glycerol, 30% glycerol/PPG, and no plasticizer were 1.01, 1.06, and 0.62 (g·mm/kPa·h·m²), respectively. It is significant to note that films without plasticizer had nearly double the water vapor permeability. This corresponded with the plasticized zein being less effective at blocking water vapor migration. Many zein plasticizers, such as glycerol, are not beneficial to zein solids because they are polar and migrate to the surface of the matrix (Parris and Coffin 1997). The plasticized zein initially is pliable, but as the glycerol bleeds to the surface, the zein becomes brittle. One plasticizer often overlooked is water. Zein plasticized with water has increased flexibility, but again becomes brittle when dehydrated, as occurs with other plasticizers (Wu et al 2003).

Fatty acids like oleic acid can be used to plasticize zein because of their interaction with non-polar amino acids, such as proline and leucine (Geraghty et al 1981, Lai and Padua 1998). Flexibility of extracted zein formed into film, after the removal of solvent most likely originates from endogenous corn pigments and lipids (Selling and Woods 2008, Parris and Dickey 2001). Parris et al (2002) found that the presence of endogenous oils could have drastic effects on tensile strengths and elongations to break of the zein films. Oleic acid was used as a plasticizer for zein to decrease water vapor permeability (Lai and Padua 1998). Wang et al (2004) characterized oleic acid/zein resins and showed a formation of structured alternating layers of zein and lipid at nanoscale that led to improved barrier properties. Wang

et al (2003) studied the binding of oleic acid to zein for plasticization using thermal conditions. They found that by extruding oleic acid/zein the resulting films had higher degree of plasticization and decreased phase separation over films without extrusion.

Another study by Wang and Padua (2006) found that oleic acid/zein films had different water vapor permeability depending on the ambient temperature. Films at 4 °C had lower water vapor permeability because of crystallized oleic acid, and films at 25 °C had higher water vapor permeability because of oleic acid in its liquid phase. Rakotonirainy and Padua (2001) studied fusion lamination and the effect of drying oils on oleic acid/zein films. Lamination produced films that were clearer, tougher, smoother, and more flexible than untreated oleic acid/zein films. Both oleic acid/zein films that had either the drying oil coating or lamination had increased tensile strengths, % elongations, and toughnesses, but had a decreased moduli. The films with either lamination or drying oils both had decreased O₂ and CO₂ permeabilities, but only drying oils decreased water vapor permeabilities.

Kleen et al (2002) found that as the oleic acid oxidized the films, they lost color, became brittle, and had off-odors. They used butylated hydroxyanisole (BHA) as the antioxidant and found that at 4,000 ppm it protected the loss of natural zein pigments in the film over the control. Wang and Padua (2004) also showed that extrusion and plasticization of zein with oleic acid reduced water adsorption over zein powder alone. Zein nanocomposites are also of interest because of their inherent ability to decrease water vapor permeability and oil permeation (Arora and Padua 2010).

2.8.2 Zein Modification and Cross-linking

Zein films are inherently water resistant, but gradual absorption of water decreases zein's utility as a packaging material. Biswas et al (2009) proposed a method to modify the surface chemistry of zein by derivatizing the film with octenyl succinic anhydride and alkyl and alkenyl ketene dimers. These compounds react with surface residues and successfully decrease the ability of the film to absorb water during immersion. Wang and Padua (2005) showed that moisture absorption of the film could be reduced through the use of drying oils, such as flax or tung oil, which can be cured on the films with UV light or γ -radiation. In a wetting test using water for 10 days, films with the oil coatings did not allow water to penetrate. Films without the oils allowed water to penetrate within one day. Compounds such as polycaprolactone (PCL) have been shown to be successful copolymers with zein to improve water-resistance (Wu et al 2003). The incorporation of PCL and plasticization with dibutyl L-tartrate in compression molded zein sheets improved water resistance, tensile strength, and elongation.

Cross-linking zein protein matrix can increase the strength and water resistance of zein films. Proteins, such as zein, have a wide variety of reactive side groups such as amide (53%), amine (1%), carboxyl (4%), hydroxyl (24%), and phenolic (8%) (Spence 1994). Many different compounds, such as formaldehyde, glutaraldehyde, and epichlorohydrin, are used to cross-link zein proteins (Parris and Coffin 1997). When each of these compounds was cross-linked with zein in aqueous ethanol, tensile strength and modulus both significantly increased over the control. The opposite was true for films produced in aqueous acetone. The increased strength of cross-linked over noncross-linked zein in ethanol solution

may be because the zein protein stays folded in solution. The steric effects of side groups do not allow the zein to efficiently align and form strong films (Yang et al 1996). Sessa et al (2007) studied the effects of cross-linking zein with glutaraldehyde in acetic acid. The zein was cross-linked with glutaraldehyde in a closed system and formed a gel that was not soluble in solvents that normally dissolved zein (Sessa et al 2007). Prior to testing, the gel bars were placed in boiling water for 10 min or 24 h in room temperature water prior to testing. The bars that were cross-linked retained their shape from both the 10 min boiling and 24 h standing in water; they showed increased strength, ductility, and stiffness compared to untreated zein samples.

Sessa et al (2008) also cross-linked zein using glutaraldehyde while compression molding at a pressure of 12500 psi and temperature of 99 °C. The bars produced with and without compression molding were similar in regards to tensile strength, ductility, and stiffness over unmodified controls. The benefit to compression molding was that it could reduce solvent use and improve recovery of acetic acid (Sessa et al 2008). The effects of time, temperature, and concentration of glutaraldehyde used for curing of electrospun zein fibers was explored by Selling et al (2008). Zein fibers that were derivitized with glutaraldehyde prior to spinning had increased tensile strength and were not soluble in standard zein solvents, while fibers produced without glutaraldehyde were still soluble. Heat promoted cross-linking, but did not improve tensile strength of the fibers.

Woods and Selling (2007, 2008) and Selling et al (2009) cross-linked zein with glyoxal using extrusion in the presence of base. Woods and Selling (2007) cross-linked zein with glyoxal, formaldehyde, and methylglyoxal. The zein bars cross-linked with

formaldehyde and glyoxal had significantly increased in tensile strength over the control and were also resistant to boiling water. Methylglyoxal did not increase the tensile strength. Woods and Selling (2008) evaluated effect of concentrations of base and glyoxal, and melt temperature on compression molded zein bars. Varying temperature and time during the melt-processing step prior to compression molding did little to change the solubilities of the zein bars. The tensile strength of the zein bars did increase with increased processing time and temperature.

Selling et al (2009) used a twin-screw extruder to cross-link zein with glyoxal during the extrusion; the bars were resistant to dissolution in acetic acid whether they were injection molded or compression molded. Compression molded samples had higher tensile strengths than those that were injection molded. They found that the incorporation of glyoxal only improved resistance to acetic acid and not tensile strength of the samples. Also, even though these cross-linking agents impart strength, many of them are toxic (e.g., formaldehyde) and may have limited practical use with zein unless they can be rinsed away or rendered inert during processing. Milder cross-linking reagents like 1-[3-dimethylaminopropyl]-3-ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide were used to cross-link zein (Kim et al 2004). These reagents are zero-order cross-linking reagents, which lose atoms during the reaction and link the carboxyl and amine groups of two different protein molecules. The cross-linking decreased aggregation and improved the tensile strength of the zein. The cross-linking agent could also be washed away with water for a resulting non-toxic zein.

It is also possible to sufficiently cross-link zein using safer compounds such as water. Pelosi (1997) mixed water and zein together and heated the mixture to 150 °C for 22 min in a

press with a total force of 4,500 kg, and cooled the mixture to 60 °C with pressure before removing the product. This product was sufficiently cross-linked to not dissolve in 50% acetic acid, which they claimed was a good indicator of complete cross-linking. However, solubility testing in 50% acetic acid may not be a good indicator as it is known that zein is readily soluble in a single solvent (primary solvent), such as acetic acid (Selling and Woods 2008), and data are lacking on whether acetic acid and water would be a good zein solvent. Most likely, an acetic acid and water mixture would not appreciably dissolve uncross-linked zein (Lawton 2002) and would not be a good indicator of cross-linking.

2.9 Zein Applications

Zein has had a variety of applications varying from plastics, coatings, inks, chewing gum, adhesives, and fibers to name a few (Simonds et al 1949, Coleman 1939, Coleman 1941, Lougovoy 1949, Sturken 1938, Croston et al 1945). When synthetic materials became cheaper in the 1950's zein products were not cost effective and lost markets. A comprehensive literature search of patents from 1891-1953 was compiled by Rathman (1954). Those seeking information on zein applications prior to 1954 should seek that source.

Currently much of the zein from CGM is used for coatings on food and pharmaceuticals (Shukla 1992). Being mostly non-polar in nature, zein films have been explored for coatings in numerous food applications. Rakotonirainy et al (2001) used 3-ply pressed oleic acid zein resin sheets laminated with tung oil for broccoli preservation. Both the zein film and polyethylene films retained broccoli firmness and color after 6 days in refrigerated storage; broccoli in only the zein films lacked off-odors. Another method used

zein to help preserve the integrity of a turkey product (Ilter et al 2008). They dusted the turkey with zein and soy protein isolate prior to frying. Zein's film forming properties were credited for helping reduce the uptake of oil during frying. Zein coatings have even been considered as a means to control the undesirable germination of seeds. Broccoli and sugar beet seeds germinated later and more slowly when dressed with a light zein coating (Assis and Leoni 2009). The slow germination was attributed to the coating preventing moisture permeation.

In recent years, the hydrophobicity of zein in water dispersions has been highly studied. Micro- and nanospheres of zein have wide variety of uses in the food and drug sector. Stark and Gross (1991) detailed the controlled production of microparticles of zein and showed that it could be used as a substitute for most dietary fats owing to lower caloric density of protein than lipids. The size of fat substitute particles was approximately 4.0 μm giving the apparent mouthfeel of fat. Micro/nanoparticles of zein have been studied as carriers of non-polar drugs; microspheres of zein have been produced that contain Ciprofloxacin, an antibiotic (Fu et al 2009). These antibiotic-laden zein spheres inhibited bacterial growth compared to control spheres.

Recently, new applications of zein have emerged for the the biomedical and controlled self-assembly fields. Many of these new processes need purified decolorized/deodorized zein. Dong et al (2003) grew human liver cells (HL-7702) and mice fibroblast cells (NIH3T3) on zein films and used polylactic acid (PLA) and Corning microplates as control. The zein films were produced from zein particles that agglomerated upon drying. The zein film with the smallest zein particles produced from the solvent (0.3%

w/v) gave the best results for proliferation of both cells after 3 days. The films produced from zein particles 100-2,500 nm in size did not show significant differences in cell proliferation. Zein is promising for tissue work because it has high tensile strength to support the cells. The film/scaffold dissolution after about 2 weeks is beneficial when replacement cells have taken hold and no longer need the support scaffold. More elaborate 3-dimensional porous zein scaffolds for tissue support produced by Wang et al (2007) had about 80% porosity with and 100-380 μm diameter pores. The zein scaffolds were implanted into 15 rabbits over a period of 242 days. The state of the scaffold degradation and tissue growth was observed at 7, 28, 91, 183, and 242 days by euthanizing rabbits. The rabbits showed good tissue compatibility; blood vessels could form within the scaffolds and the scaffolds were completely degraded at the end of 242 days.

Tu et al (2009) studied the growth of bone tissues on zein scaffolds to repair critical bone damage to the radius bone of the rabbit. They studied the bone repair with X-ray imaging of control without assistance, repair with zein scaffold, and repair with zein scaffold and rabbit mesenchymal stem cells (MSC) at 2-12 weeks. It was apparent through gross observation at the end of 12 weeks that the control bone was still highly damaged, the zein scaffold supported bone was partially repaired, and the bone with scaffold and MSC was nearly repaired.

Electrospun zein fibers cross-linked with citric acid were prepared by Jiang et al (2010) for growth with NIH 3T3 mouse fibroblast cells. The fibers were prepared as mats and treated with phosphate-buffered saline (1xPBS, pH 7.4). They analyzed the growth of cells on electrospun zein scaffolds that were cross-linked and treated with PBS, electrospun

uncross-linked zein scaffolds, electrospun cross-linked zein scaffolds with sodium hypophosphite monohydrate (SHP), and electrospun PLA. Zein scaffolds that were cross-linked and treated with PBS grew cells that had the best attachment, spreading, and proliferation. The zein fiber scaffolds supported cell proliferation better than film based zein scaffolds because of higher porosity in mats (Jiang et al 2010). The observation of higher cell growth with higher porosity is consistent with the results of Dong et al (2003).

Zein scaffolds, have also been produced to coat on surfaces by taking advantage of its chemical affinities (Wang et al 2008). They showed that by patterning certain hydrophobic or hydrophilic compounds on gold sheets, and allowing a zein solution to self-assemble on them produced zein overlays on these patterns, which interacted differently based on the compounds' water affinity. This ability to produce controlled zein structures would be very important for the consistent production of highly ordered zein scaffolds.

2.10 Summary

Zein is a protein biopolymer that is renewable and can be extracted from corn and corn co-products. The ability for zein to be renewable is important now that other synthetic polymers are tied to increasing prices in oil. Also, enhancing zein's importance is its inherent water insolubility and ability to be plasticized and cross-linked, which can impart desired flexibility, strength, toughness, permeation resistance, and solvent insolubility. New interest in utilizing renewable polymers has helped spur interest in zein extractions and applications. As more corn becomes used in the dry-grind ethanol process, zein extraction from both front- and back-end co-products have been proposed. The current commercial zein protein extraction from corn gluten meal yields lower product recovery. Newer solvent

systems and extraction modifications could enhance extraction efficiency and recovery of zein both from CGM and DDGS. The subsequent chapters deal with improving the extraction of zein from CGM and DDGS. Chapter 2 focuses on using a new method and different solvent and their abilities to extract zein from CGM. Chapter 3 focuses on the extraction of zein from DDGS using the best solvents and the new method.

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CHAPTER 3. NOVEL EXTRACTION METHOD FOR α -ZEIN FROM CORN GLUTEN
MEAL USING DIFFERENT SOLVENTS

A paper submitted for publication to *Cereal Chemistry*.

by

Timothy J. Anderson¹ and Buddhi P. Lamsal² *

¹Graduate Student, Department of Food Science and Human Nutrition

²Assistant Professor, Department of Food Science and Human Nutrition

Iowa State University, Ames, IA, 50011

* Corresponding author, Email: lamsal@iastate.edu; Ph: (515) 294-8681; FAX: (515) 294-8181.

3.1 Abstract

A modified procedure for α -zein extraction from corn gluten meal was developed and compared against the commercial zein extraction method. The modified method consisted zein extraction, precipitation of most of the non- α -zein fractions, collection of the soluble α -zein and its cold precipitation from solution and drying. Five organic solvent mixtures were compared using the modified extraction procedure along with the reductant sodium bisulfite and sodium hydroxide (NaOH). The modified procedure precipitated most of the non- α -zein proteins by increasing the concentration of alcohol. After cold precipitation of α -zein from solution, the subsequent α -zein-rich solids had a higher yield of α -zein than the commercial method. The commercial extraction procedure had a zein yield of 23% and protein purity of 28% using 88% 2-propanol solvent. The three best solvents using the new extraction procedure, 70% 2-propanol, 55% 2-propanol, and 70% ethanol had zein yields of approximately 35% and protein purity of 44%, respectively. The zeins extracted using the novel method were lighter in color than those from the commercial method. Gel densitometry scans of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of α -zein-rich solids showed relatively large quantities of α -zein with apparent molecular weights of 19,000 to 22,000 Da. The α -zein-rich solids appropriately had small amount of δ -zein (10,000 Da) since it shared similar solubility properties to α -zein. A solvent with 70% 2-propanol, 22.5% glycerol, and 7.5% water extracted significantly less zein ($\sim 1/3^{\text{rd}}$) compared to all other solvents.

3.2 Introduction

Zein comprises up to 52% of the protein by weight in the corn kernel. Zein was first described by Gorham (1821) when he extracted the protein from Indian corn. Interest in the protein developed when Osborne (1891) extracted zein from corn gluten meal (CGM), a high-protein co-product of corn wet milling. Later, Osborne and Mendel (1914) classified corn protein into four different categories based on their solubilities. These proteins were albumins (soluble in pure water), globulins (soluble in aqueous salt solutions), prolamins (soluble in 70% ethanol), and glutelins (soluble in dilute acid or base). Zein proteins are prolamins and have been further characterized into four different classes based on solubility, electrophoresis, and immunological studies. Esen (1987, 1990) classified them as α -, β -, γ -, and δ -zein. Important past applications of zein were in inks, adhesives, coatings, plastics, and chewing gums (Coleman 1941, Sturken 1938, Coleman 1939, Simonds et al 1949, Lougovoy 1949). New potential applications of zein include packaging, carrier material, biomedicine e.g., zein for cellular scaffolds to accelerate cell growth in tissue and bone, while degrading after healing (Dong et al 2004, Wang et al 2007, Tu et al 2009, Jiang et al 2010). It is important to recover more functional zein fractions with higher purity to suit potential novel applications.

The first commercial extraction of zein was in 1939 (Shukla and Cheryan 2001). A refined patent detailed the commercial extraction process for zein using 85% aqueous 2-propanol at 60 °C (Swallen 1942). The extract was then treated with hexane to remove pigment, and the zein was collected by precipitating in cold water and drying in ring dryers. A current commercial method extracts zein from CGM with a solvent of 88% (w/w) 2-

propanol at 65 °C with agitation (Carter and Reck 1970). The extract is cold-precipitated at -10 to -20 °C and the precipitate is dried in a vacuum oven. To produce higher purity zein protein, the cold-precipitated wet solids could be redissolved in the extracting solvent and re-precipitated. Another commercial extraction of zein was described by Takahashi and Yanai (1994). They extracted α -zein from CGM using 70% (v/v) aqueous acetone at 40 °C. The supernatant was then concentrated and added into absolute acetone to precipitate the α -zein protein.

These current commercial extraction methods extract primarily α -zein, which is soluble in aqueous alcohol solvents with higher concentrations of alcohol, e.g., 88% (w/w) 2-propanol (Kale et al 2007, Esen 1987). A major problem with these methods is low zein yields of only about 22 and 20.4 g/g CGM (dry basis), respectively. These yields are low considering that over 50% of the α -zein remains unextracted (Wu et al 1997). Zein extraction yields could be improved by using lower aqueous alcohol concentrations, e.g., 55% (w/w) 2-propanol. This increase of yield would be due to co-extraction of all zein fractions resulting in lower α -zein purity (Esen 1986). However, lower concentrations decrease zein solubility and solution stability (Swallen 1942). Other issues with both types of extractions are a) large amounts of solvents required, and b) energy intensive processes such as solvent concentration, cold-precipitation, and distillation of solvent for recycling.

The purpose of this research was to produce a modification to the Carter and Reck (1970) zein extraction procedure that improved the yield and purity of the zein protein using a reductant and different solvents. The solvents used were selected based on known information about the structure of zein protein bodies and their solubilities. The zein protein

body consists of a thin layer of β - and γ -zein molecules interconnected via disulfide bonds. This layer sheaths a large proportion of the α -zein at the particle's core (Mohammad and Esen 1990). The commercial extraction method uses 88% (w/w) aqueous 2-propanol, no reducing agent, and 0.25% NaOH (Carter and Reck 1970). The 88% (w/w) aqueous 2-propanol dissolves α -zein across the layer without dissolving the β - and γ -zeins (Carter and Reck 1970). For this research, solvents that extract total zein, such as 55% (w/w) aqueous 2-propanol and 70% (v/v) aqueous ethanol were chosen. These two solvents with the aid of reducing agent are known to disrupt β - and γ -zeins in the protein body and are more efficient in extracting α -zein (Shukla et al 2000, Landry et al 1983). The solvent 70% (w/w) aqueous 2-propanol was chosen to determine if dissolution of β - and γ -zeins at modest levels could disrupt the protein bodies and in turn extract more α -zein. The mixture of 70% (w/w/w) aqueous 2-propanol, 22.5% glycerol, and 7.5% water was a new ternary solvent designed to dissolve zein; it was to evaluate the potential of inclusion of a co-product of biodiesel, namely crude glycerol at 30% (w/w) glycerol/water level. The objectives of this study were to 1) develop an extraction procedure to extract all zeins and compare the zein yields to the commercial method and 2) evaluate the efficacy of reducing agent sodium bisulfite in increasing zein yield.

3.3 Materials and Methods

3.3.1 Corn Gluten Meal

The CGM used in the zein extraction was obtained from Cargill Inc. (Eddyville, IA). The particle size distribution of CGM was determined by using a Malvern MasterSizer laser diffraction size analyzer (Model 2000, Malvern Instruments Ltd., Malvern, UK). The

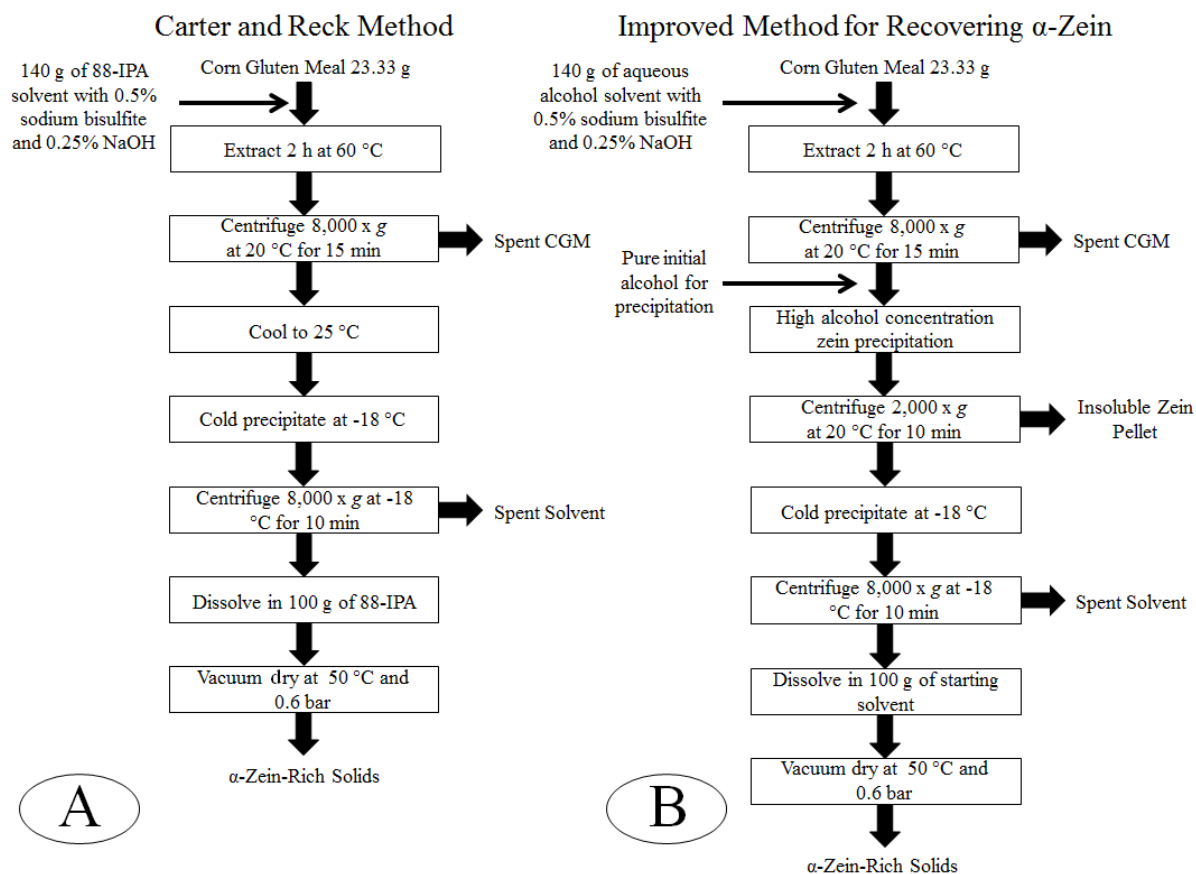
relative proportion of different types of protein in CGM was determined following the method described by Wu et al (1997). Briefly, 10 g (db) of CGM in duplicate was first extracted with 250 mL of 0.5 M sodium chloride solution in a 400-mL sealed centrifuge bottle for 20 min at room temperature with stirring to recover saline-soluble proteins. It was then centrifuged at $8,000 \times g$ for 15 min and the supernatant collected. The zein proteins were then extracted from the pellet with 250 mL of 55% (v/v) aqueous 2-propanol and 5% (v/v) 2-mercaptoethanol with 0.5% (w/v) sodium acetate solvent (PMA). The solution was stirred for 2 h at room temperature and then centrifuged at $8,000 \times g$ for 15 min. The remaining solids were washed twice with 50 mL of PMA solvent and these washes combined with the first PMA extract. Prior to protein analysis, the three fractions of saline- soluble proteins, total zein fractions, and spent CGM, were measured for total solids based on total zein extraction data from the work of Wu et al (1997). The protein content of these three fractions was determined by using the Dumas's combustion method.

3.3.2 Extraction of Zein and Comparison of Solvent Systems

Two methods of extraction were employed as outlined in Fig. 3-1: Carter and Reck's (1970) method (Method A) using 88% (w/w) aqueous 2-propanol (88-IPA), and a lab-modified Method B. Method B added a precipitation step to Method A. The following solvents were compared for extraction efficiency using Method B: 70% (w/w) aqueous 2-propanol (70-IPA), 55% (w/w) aqueous 2-propanol (55-IPA), 70% (w/w/w) aqueous 2-propanol, 22.5% glycerol, and 7.5% water (70-GLY-IPA), 70% (v/v) aqueous ethanol (70-EtOH), and 70% (v/v) aqueous ethanol with two cold precipitations (70-TCP-EtOH). Both Methods A and B extracted zein from 23.33 g of CGM (db) using 140 g of solvent in a

solute-to-solvent ratio of 1:6 at 60 °C for 1 h. Sodium hydroxide, 0.25%, and the reductant sodium bisulfite (0.5%) were added to 140 g of solvent for reductant treatment during some extractions. The zein extraction method of Carter and Reck (1970) also used 0.25% NaOH

Figure 3-1



Method A is Carter and Reck (1970) extraction method (A), Method B is a modification of Method A evaluated in this study with different solvents (B). Method B adds a precipitation and centrifuge step after crude zein extraction.

to adjust the CGM extract to pH 6.5 to 7.0, which is near the isoelectric point of many zein phenotypes causing them to stay insoluble while leaving α-zein soluble (Carter and Reck 1970, Cook et al 1996). The control extraction had no NaOH and sodium bisulfite treatment.

After the crude zein extraction following Method A, the solution was centrifuged at room temperature for 15 min at $8,000 \times g$. The extracted crude zein was decanted from the spent CGM solids and a sample of 7.5 g of crude zein extract was taken to measure solids content. The remaining extract was left at $-20\text{ }^{\circ}\text{C}$ overnight to precipitate zein protein into a taffy-like layer. This material was then centrifuged at $-20\text{ }^{\circ}\text{C}$ and $8,000 \times g$ to remove the supernatant. The zein pellet was dissolved in 100 g of 88% (w/w) aqueous 2-propanol and dried in a vacuum oven at $50\text{ }^{\circ}\text{C}$ at 0.6 bar pressure. The dry α -zein-rich solid was weighed, ground, and stored at $4\text{ }^{\circ}\text{C}$ until use. A portion of ground fraction was further dried in a vacuum oven at the same temperature and pressure for 4 days for moisture determination.

Method B followed the same crude zein extraction step as in Method A but using solvents 70-IPA, 55-IPA, 70-GLY-IPA, 70-EtOH, or 70-TCP-EtOH. After extraction, 2-propanol was added to achieve 88% (w/w) aqueous concentration to the extracts obtained with 70-IPA, 55-IPA, and 70-GLY-IPA. For the supernatant obtained with 70-EtOH and 70-TCP-EtOH, alcohol concentrations were increased to 95% (v/v) aqueous ethanol. The alcohol was added slowly and continuously. This step precipitated β - and γ -zeins leaving α -zein in solution (Esen 1986, Parris and Dickey 2001). The resulting turbid solution was then stirred for 30 min, centrifuged at $2,000 \times g$ for 10 min at room temperature. The protein pellet was dried, weighted, and analyzed for protein content. Since the extraction was performed in sealed centrifuge tubes, the solvent concentration was assumed to remain constant during extraction. The amount of alcohol used to increase the solvent concentrations to precipitate β - and γ -zeins was based on the mass of estimated crude zein in the extract from the total zein extractions of Wu et al (1997). The clear supernatant was held

at -20 °C overnight to allow protein precipitation. The precipitate was centrifuged at $8,000 \times g$ at -20 °C for 15 min, the supernatant discarded. The α -zein-rich solid was dissolved in 100 g of either 88% (w/w) aqueous 2-propanol or 95% (v/v) aqueous ethanol based on which alcohol was used for the primary extraction. The dissolved α -zein was placed in a 600-mL beaker and dried in the vacuum oven at 50 °C at 0.6 bar (Wu et al 1997). When dried, the α -zein-rich solids was weighed, ground, and stored at 4 °C until use. A portion of ground sample was further dried in the vacuum oven at the same temperature and pressure for 4 days for moisture determination.

3.3.3 Extraction Parameters and Calculations

The extraction terminologies differed for various measured extraction parameters. The term *crude zein* indicated the recovered protein during the initial extraction of CGM. *Crude zein protein, %* = $[(\text{mass protein extracted})/(\text{total protein in CGM mass})] \times 100$. The remaining solids after crude zein extraction was termed *spent CGM*. *Spent CGM protein, %* = $[(\text{mass protein unextracted})/(\text{total protein in CGM mass})] \times 100$. In Method B, the insoluble *zein pellet* was precipitated from the alcohol extract of crude zein, leaving the α -zein-rich proteins in solution. The *insoluble zein pellet* = $[(\text{mass protein precipitate in pellet})/(\text{total protein in CGM mass})] \times 100$.

Zein yield was the mass of zein extracted relative to the mass of starting material. *Zein yield, %* = $[(\text{mass } \alpha\text{-zein-rich solids})/(\text{total mass of CGM (db)})] \times 100$. The *protein purity* is the protein content of the recovered α -zein-rich solid. The *protein recovery* is a more accurate final yield parameter for zein taking into account the percent protein of the α -zein-rich solids relative to the mass of protein in the initial substrate (Wu et al 1997). *Protein*

recovery, % = (*protein purity, %*) × (mass α -zein-rich solids)/(total mass of protein in starting CGM). The α -zein extraction efficiency determined the amount of α -zein recovered at the end of the extraction. α -Zein extraction efficiency, % = (*protein purity, %*) × (mass of α -zein-rich solids, %)/(total mass of α -zein protein in starting CGM). The fraction of α -zein relative to the total zein protein in CGM was determined by using densitometry as described below (Section 3.3.5).

3.3.4 Composition Analysis

The moisture content of the CGM was determined by drying samples at 130 °C for 3 h in a convection oven following AACC Method 44-19 (AACC 2000b). The solids content of spent CGM and γ -zein pellet samples were measured by oven drying overnight at 103 °C (Dickey et al 1997). Crude free fat contents were determined by using AACC method 30-25 with the Goldfish apparatus (Labconco Corp., Kansas City, MO) and hexane as solvent. All the analyses were conducted in duplicate and data was expressed on moisture-free basis. Crude protein contents were determined by using the Dumas nitrogen combustion method with an Elementar Vario MAX CN analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) according to AOAC Method 992.23 (AOAC 1998). The conversion factor used to estimate protein was $6.25 \times$ nitrogen.

3.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Densitometry

The α -zein-rich solids and the total zein samples per Wu et al (1997) were separately analyzed by using SDS-PAGE and densitometry. The SDS-PAGE gels were prepared

according to methods of Laemmli (1970). Because of the non-polar characteristics of zein, it was difficult to dissolve α -zein-rich solids in polar sample buffers. To disperse and homogenize the zein protein, the solid was first dissolved in 70% (v/v) aqueous ethanol and then added to a sample buffer consisting of 3.55 mL deionized water, 1.25 mL of 0.5 M Tris HCl, pH 6.8 buffer (6.055% aqueous solution of tris(hydroxymethyl)aminomethane and 0.4% SDS), 2.5 mL glycerol, 2.0 mL of a 10% (w/v) SDS solution, 0.2 mL of a 0.5% (w/v) bromophenol blue solution, and 0.5 mL of 2-mercaptoethanol. The dissolved zein to sample buffer ratio was kept <0.5 to keep samples from migrating out of the well due to buoyancy. After the dissolved zein was added to the sample buffer the mixture was heated at 100 °C for 5 min and left at 4 °C until applied to the gel. Stacking gels and the resolution gel were prepared at 4 and 13% acrylamide, respectively. For the total zein sample, the same stacking gel concentration was used, but the resolution gel was increased to 15% acrylamide to better resolve the proteins. The α -zein-rich sample (40 μ L) was loaded at a concentration of 3 μ g/ μ L to the gels. The protein standard used was a low molecular weight marker (Sigma M-3913, St. Louis, MO) consisting of proteins with MW's from 6,500 to 66,000 Da. SDS gel images were scanned using a Biotech image scanner (Amersham Pharmacia, Piscataway, NJ). Densitometry was performed using ImageJ software developed at the National Institutes of Health. Calculations for densitometry were as follows: *Compositon, %* = [(band or sum of subunit bands)/ (all bands measured)] \times 100.

3.3.6 Statistical Analysis

For the zein extraction experiment, six different solvent extractions were compared; each solvent run with or without NaOH (0.25%) and the reductant sodium bisulfite (0.5%).

The 12 treatments were run as a randomized complete block design, replicated twice, and the data analyzed using ANOVA and Tukey, $p < 0.05$ for significance using JMP v. 8.0.1 statistical software (SAS Institute, Inc., 2010).

3.4 Results and Discussion

3.4.1 CGM and Total Zein in CGM

The CGM used for extraction contained 9.2% moisture, 69.6% protein (db), and 2.9% hexane-extractable lipids (db). The particle size distribution of CGM ranged from 3 to 700 μm . The median particle diameter, d_{50} , was about 100 μm and used for extraction without modification. The protein contents of CGM for saline-soluble, total zein, and spent CGM were 2, 44, and 21%, respectively. While only a small fraction (2%) of the total protein was saline-soluble protein, a large amount of protein (21%) was completely insoluble and remained with the solid residue. The sum of the protein percentages observed in three CGM fractions was 67%, which compared well to 70% total protein in CGM (db) measured by Dumas's combustion method.

3.4.2 Extraction of Zein Proteins and Protein Balance

Solute-to-solvent ratio played a role in zein extraction; preliminary extractions with all six solvents were performed following methods of Carter and Reck (1970) at 1:4 solute-to-solvent ratio (data not shown). Solvents, such as 70-IPA, 55-IPA, and 70-EtOH, extracted total zein proteins and the extracts were highly concentrated and had poor solution stability similar to zein solutions described in Swallen (1942) and Pomes (1971). The poor stability caused the protein to gel and precipitate out of solution with spent CGM decreasing yield. At

higher solute-to-solvent ratios, the gelling decreased; the zeins were then extracted at the 1:6 solute-to-solvent ratios for all extractions.

The proteins recovered in the crude zein extract and remaining in the spent CGM after extraction with different solvents and treatments are presented in Table 3-1. There was no significant difference ($p < 0.05$) between the extractions with or without reductant using 88-IPA following Method A; however, 88-IPA extracted significantly less protein than all the solvents following Method B. The solvents that extracted the most protein from CGM as crude zein were 55-IPA, 70-EtOH, and 70-TCP-EtOH with reductant following Method B. These solvents extracted >30% more zein protein than 88-IPA following Method A. Without the presence of the reductant, these three solvents extracted about 20% more protein than 88-IPA, with 55-IPA extracting slightly more protein than the other two solvents. This was because the γ -zein was further reduced and dissolved with the aid of the reductant and low alcohol concentrations. With the steeping in the corn wet-milling, many of the disulfide bonds connecting the proteins on the outer layer of the protein body are severed making the γ -zein extractable even without the benefit of reductant during zein extraction (Esen 1987, Neumann 1987). The sums of the protein contents in crude zein and spent CGM were nearly 100% for all of the extractions (Table 3-1), except 70-GLY-IPA for extraction with or without reductant. During the measurement of solid content in crude zein from 70-GLY-IPA, there was syneresis of glycerol from the solid matrix. This remaining glycerol could not be homogenized into the sample prior to protein analysis, thus, creating solids containing erroneously higher amounts of protein.

The protein in insoluble zein pellet (Table 3-1) represented the amounts of protein

Table 3-1

Percentage of CGM Protein in Extract Fractions with Solvent Treatments

Treatment	Solvent System	Crude Zein^b, %	Spent CGM^c, %	Insoluble Zein Pellet^d, %	Protein Recovery^e, %
Reductant and NaOH	88% 2-Propanol	33.4 d	69.1 a	NA	27.9 c
	70% 2-Propanol	59.7 ab	38.6 de	9.0 d	44.8 a
	55% 2-Propanol	65.0 a	33.5 f	14.2 c	43.2 a
	70% 2-Propanol w/glycerol	57.2 ab	58.3 b	25.0 a	9.7 d
	70% Ethanol	64.5 a	33.7 ef	13.7 c	44.8 a
	70% Ethanol two precipitations	64.0 a	34.7 ef	13.2 c	43.4 a
No Reductant and No NaOH	88% 2-Propanol	31.3 d	70.0 a	NA	26.7 c
	70% 2-Propanol	54.7 b	44.2 c	10.6 d	37.6 b
	55% 2-Propanol	57.8 ab	40.4 cd	13.8 c	36.7 b
	70% 2-Propanol w/glycerol	44.0 c	67.2 a	20.7 b	7.2 d
	70% Ethanol	54.4 b	44.0 c	20.2 b	28.1 c
	70% Ethanol two precipitations	54.4 b	43.4 cd	20.7 b	28.5 c

^a Values Followed by different letters within the same column indicate significant differences ($p < 0.05$).

^b Crude zein protein = $[(\text{mass protein extracted})/(\text{total protein in CGM mass})] \times 100\%$

^c Spent CGM protein = $[(\text{mass protein unextracted})/(\text{total protein in CGM mass})] \times 100\%$

^d Insoluble zein pellet = $[(\text{mass protein precipitate in pellet})/(\text{total protein in CGM mass})] \times 100\%$

^e Protein recovery = $[((\text{protein purity, \%}) \times (\text{mass } \alpha\text{-zein-rich solids})) / (\text{total protein in CGM mass})]$

lost in the pellet during purification of α -zein when the solution concentration increased to 88% (w/w) 2-propanol or 95% (v/v) ethanol. For 70-IPA and 55-IPA, the presence of reductant did not significantly change the amount of protein precipitated, but there was significantly more protein precipitated with the pellet without the presence of reductant for 70-EtOH and 70-TCP-EtOH. Without the aid of a reducing agent with 70-EtOH and 70-TCP-EtOH, a small fraction of α -zein must precipitate out of solution with γ -zein to account for the increase of mass for the insoluble zein pellet. In a similar extraction from CGM using 60% (v/v) 2-propanol containing 1% β -mercaptoethanol, Parris and Dickey (2001) showed that as much as 78% of the γ -zein pellet was α -zein, 12% γ -zein, and 5% an unknown protein band (14,000 Da). The process by which α -zein precipitates out of solution with γ -zein, even while in a solvent in which α -zein is soluble, is not understood. 70-GLY-IPA lost more of protein in the insoluble zein pellet showing that a reducing agent did not behave the same in reducing the pellet mass in the presence of glycerol.

The protein recoveries of α -zein extracted with 70-IPA, 55-IPA, 70-EtOH, and 70-TCP-EtOH with reductant were the highest with no significant difference between their yields (Table 3-1). However, all four solvents showed a significant difference over the same solvents without reductant. This significantly higher yield for the four solvents with reductant was probably due to the reduction of the remaining interconnected γ -zein, allowing better solubility of all zein. Also, the reductant decreased the amount of protein precipitated in the γ -zein pellet increasing the final yield of zein due to better extraction and less zein precipitation. Both 88-IPA and 70-GLY-IPA had no difference in yield regardless of reductant use. For 88-IPA, this probably could be because α -zein is readily extractable

without reducing agent. Small differences in yield were observed for 70-GLY-IPA with or without reductant.

The protein recovery accounted for the portion of protein recovered based on the protein fractions in the initial CGM (Wu et al 1997). Wu et al (1997) extracted zein from CGM with a method similar to Method A and their highest protein recovery was 32%. This recovery yield is nearly 12% less than that obtained with 70-IPA, 50-IPA, 70-EtOH, or 70-TCP-EtOH using Method B in the presence of reductant, and less than for solvents 70-IPA and 55-IPA without reductant. The solvent 70-GLY-IPA extracted the least amount of α -zein regardless of the presence of reductant.

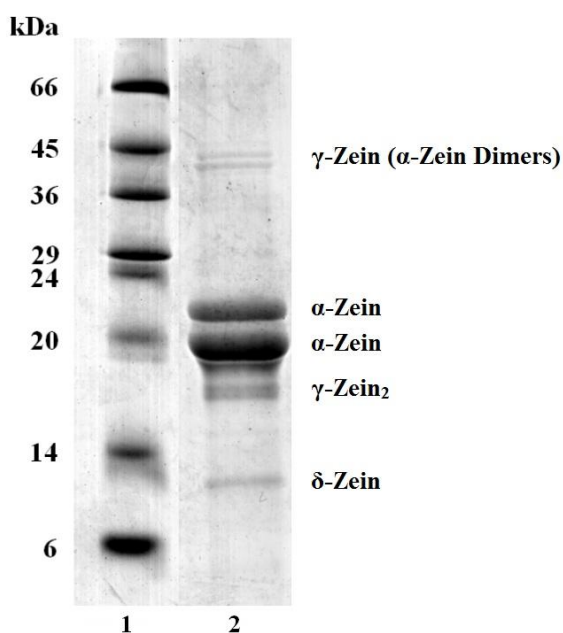
3.4.3 SDS-PAGE and Densitometry

The SDS-PAGE of total zein which was extracted following the method of Wu et al (1997) is presented in Fig. 3-2. Using the nomenclature of Esen (1987, 1990), total zein contains mostly α -zein with MW's of 19,000 and 22,000 Da, γ_2 -zein at 18,000 Da, δ -zein at 10,000 Da, and dimers of α -zein near 45,000 Da. The densitometry scan of the SDS-PAGE gel of total zein (Fig. 3-2) indicated that 65% of the fraction was α -zein, 13% γ_2 -zein, 9% δ -zein, and 12% dimers of α -zein. The γ_1 -zein band at MW 28,000 Da was not observed in the gel, just as reported by Parris and Dickey (2001). They attributed the disappearance of γ_1 -zein to cleavage and loss during the steeping of CGM. Also missing was the β -zein band, which is a protein at 14,000 Da (Wilson 1991).

The molecular bands of α -zein-rich solids obtained using different extraction solvents with or without reductant are presented in Fig. 3-3. The relative purities of the α -zein-rich

solids were determined with these SDS-PAGE gels. There were little differences in the α -zein bands with or without the reductant. The SDS-PAGE of total zein (Fig. 3-2) showed that it contained proteins which were not observed in the other α -zein-rich solids extracted (Fig. 3-3).

Figure 3-2

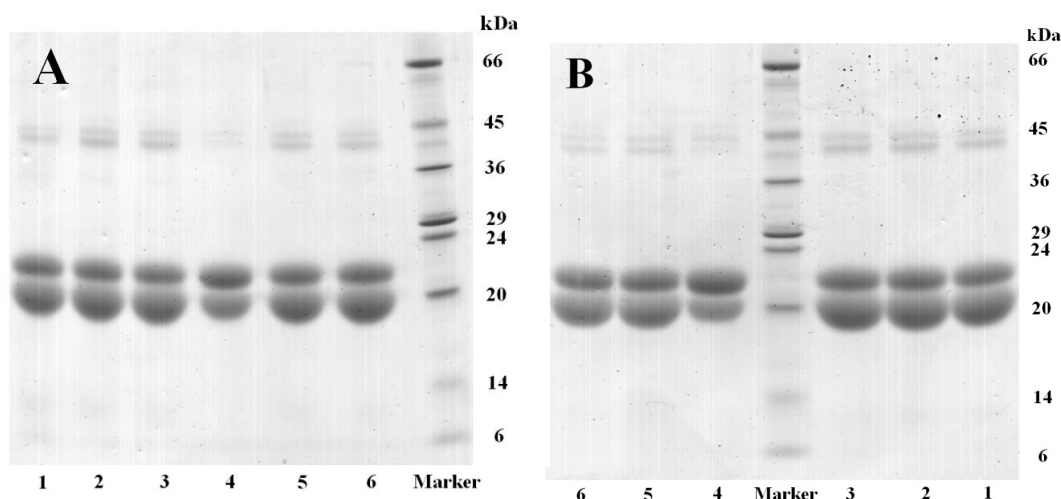


Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total zein extract. Lane 1 is marker and lane 2 is total zein protein fraction based on the extraction method of Wu et al (1997).

When considering the protein bands in both the total extracted zein (Fig. 3-2) and α -zein-rich solids (Fig. 3-3), the amount of extractable α -zein-rich solids was 87% of the zein protein. Taking this into account, the α -zein-rich solids yield was 38% of the mass of the CGM based on 44% of the protein being extractable zein. Faint bands at MW 10,000 Da were present in the α -zein-rich solids from 88-IPA, 70-IPA, 55-IPA, 70-EtOH, and 70-TCP-EtOH with and without reductant, which indicates the presence of some δ -zein in these

extracts. The extract using 70-GLY-IPA had α -zein bands different than the other five extracts. The two bands at 19,000 and 22,000 Da had different shapes than the other zein extracts. The 19,000 Da band was less intense while the 22,000 Da band was more intense. It is not known if this would affect the properties of the extracted α -zein or if there were different amounts of minor α -zein proteins in the zein extracted with 70-GLY-IPA.

Figure 3-3



SDS-PAGE of α -zein extracts. Gel A is comprised of α -zein-rich solids extracted with NaOH and the reductant sodium bisulfite and gel B α -zein fractions were extracted without reductant and NaOH. The α -zein extracted using different solvents: 88% 2-propanol (lane 1), 70% 2-propanol (lane 2), 55% 2-propanol (lane 3), 70% aqueous 2-propanol, 22.5% glycerol, and 7.5% water (lane 4), 70% ethanol with one cold precipitation (lane 5), 70% ethanol with two cold precipitations (lane 6).

3.4.4 α -Zein Extraction Efficiency and Yields

The α -zein extraction efficiencies, and the protein purities for the solvent systems based on densitometry, are presented in Table 3-2. α -Zein extraction efficiency decreased in absence of reductant; prior reduction of zein disulfide bonds during steeping and partial oxidization of disulfide bonds during the drying of CGM (Neumann et al 1987) could be one

reason. The γ -zein proteins that are interconnected through disulfide binding must become fully reduced when exposed to the reductant to allow full dissolution of zein (Esen 1987). Extraction with 70-IPA, 55-IPA, 70-EtOH, and 70-TCP-EtOH had α -zein efficiencies nearly 30% higher than 88-IPA with reductant.

The zein yield was included in Table 3-2 to compare against literature data, but zein yield has been a poor method of reporting yield. The zein yield does not compensate for non-protein impurities, which could constitute as much as 40-80% of the solid contents in zein extracts (Shukla et al 2000). Bound/trapped moisture is a non-protein parameter that greatly affects zein yield. To determine moisture content in the α -zein-rich solids, they were further ground and dried in a vacuum oven to complete dryness. All samples contained 4-5% moisture indicating that the true protein purities (db) could be higher than values in Table 3-2

3.5 Conclusions

The solvents 70-IPA, 55-IPA, and 70-EtOH extracted significantly more α -zein-rich solids from CGM when using Method B compared to commercial method (Method A). Sodium bisulfite and NaOH enhanced zein yields. Dispersal of non-protein impurities in more dilute aqueous alcohol solvents caused the subsequent cold-precipitated α -zein-rich solids to entrap fewer impurities, thereby having higher purity. This has implications in eliminating double cold-precipitation process that is utilized to increase purity of zein proteins. The significance of the present research lies in the fact that a modified zein extraction method was devised and shown to extract more zein from CGM substrate.

Table 3-2

Zein Yields, Zein Extraction Efficiencies, and Protein Purities

Treatment	Solvent Systems	α -Zein		
		Protein Purity ^b , %	Extraction Efficiency ^c , %	Zein Yield ^d , %
Reductant and NaOH	88% 2-Propanol	83.2 c	51.2 c	23.4 c
	70% 2-Propanol	87.6 bc	82.1 a	35.6 a
	55% 2-Propanol	88.6 abc	79.1 a	33.9 a
	70% 2-Propanol w/glycerol	63.4 d	17.8 d	10.7 d
	70% Ethanol	91.1 abc	82.1 a	34.3 a
	70% Ethanol two precipitations	94.0 ab	79.6 a	32.2 ab
No Reductant and No NaOH	88% 2-Propanol	86.5 bc	49.0 c	21.5 c
	70% 2-Propanol	88.1 abc	68.8 b	29.7 b
	55% 2-Propanol	88.2 abc	67.3 b	29.0 b
	70% 2-Propanol w/glycerol	64.5 d	13.2 d	7.8 d
	70% Ethanol	90.7 abc	51.5 c	21.6 c
	70% Ethanol two precipitations	96.1 a	52.3 c	20.7 c

^aValues Followed by different letters within the same column indicate significant differences ($p < 0.05$).

^bThe protein purity is the protein content of the recovered α -zein-rich solids

^c α -Zein extraction efficiency = $[(\text{protein purity, \%}) \times (\text{mass } \alpha\text{-zein-rich solids}) / (\text{total mass of } \alpha\text{-zein protein in CGM})]$

^dZein yield = $[(\text{mass } \alpha\text{-zein-rich solids}) / (\text{total mass of CGM (db)})] \times 100\%$

It remains to be seen how the modified method performs with substrates that vary in quantity and quality of zein present in the starting material for example, DDGS from various dry-grind ethanol processes.

3.6 Acknowledgements

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CHAPTER 4. IMPROVED α -ZEIN EXTRACTION FROM DISTILLERS' DRIED
GRAINS WITH SOLUBLES AND CHARACTERIZATION

A paper to be submitted to *Industrial Crops and Products*.

Timothy J. Anderson¹, Ilankovan Paraman², and Buddhi P. Lamsal³*

¹Graduate Student, Department of Food Science and Human Nutrition

²Postdoctoral Research Associate, Department of Food Science and Human Nutrition

(Co-author Ilankovan Paraman assisted with preparation and characterization of the zein
films, and with edits of draft manuscript)

³Assistant Professor, Department of Food Science and Human Nutrition

Iowa State University, Ames IA, 50011

* Corresponding author, Email: lamsal@iastate.edu; Ph: (515) 294-8681; FAX: (515) 294-8181.

4.1 Abstract

Zein was extracted from distillers' dried grains with solubles (DDGS) using three solvents and two different extraction methods (commercial, and a modified). Control solvent 88% (w/w) aqueous 2-propanol, and two other solvents, 70% (w/w) aqueous 2-propanol and 70% (v/v) aqueous ethanol were compared for their extraction of α -zein-rich solids from DDGS following the modified procedure. Recovery parameters compared were extraction yield, purity, and zein film characteristics of recovered zein. Efficacy of cellulase treatment and DDGS size reduction was evaluated for their increase in zein extraction. The protein recovery values for 70% (w/w) aqueous 2-propanol, 70% (v/v) aqueous ethanol, and 88% (w/w) aqueous 2-propanol were 13.7, 13.5, and 2.5%, respectively; this showed that the modified method extracted significantly more zein compared to commercial method. The enzyme treatment had no effect on increasing zein extraction yield, but reduced particle size of DDGS increased zein yield. SDS-PAGE of the DDGS extract showed bands with MW of about 40,000, 22,000, 19,000, and 10,000 Da, which corresponded to α -zein dimers, α -zein (Z22), α -zein (Z19), and δ -zein, respectively. The total zein extracted with 55% (v/v) aqueous 2-propanol and 5% (v/v) 2-mercaptoethanol with 0.5% (w/v) sodium acetate, in contrast, contained high amounts of α -zein, but substantial amounts of γ_1 - and γ_2 -zein also were present. The α -zein films extracted from DDGS without enzyme treatment were cloudy with rough surfaces, unlike the glossy and smooth films formed from α -zein extracted from CGM and enzyme treated DDGS.

4.2 Keywords: Corn, proteins, zein, DDGS, protein extraction, prolamins, ethanol.

4.3 Introduction

Corn zein is comprised of α -zein, β -zein, γ -zein, and δ -zein fractions, based on zein solubility in 2-propanol (Esen 1987, 1990). The α - and δ -zein are found in the protein body core, while β - and γ -zein are on the periphery of the protein body (Thompson and Larkings 1989, Mohammad and Esen 1990); however, α - and δ -zein are the only zeins considered true prolamins. β - and γ -zein are considered glutelins based Osborne's solubility principles, but are considered zeins because of their inclusion within zein protein bodies.

Zein can be extracted from three different corn materials: dry-milled corn (DMC), CGM, and DDGS. The least processed material is DMC, which can contain approximately 6.8-8.0% protein, of which 52% are considered zein proteins (Rausch et al 2009). Some corn hybrids have higher dry-milled endosperm fractions with protein concentrations as high as 18.7% (Wolf et al 1975). The extraction of zein from DMC is uneconomical because of the large amounts of solvent needed and low extraction yields. CGM, another product of the corn wet-milling industry, is typically used to extract zein commercially, as it contains 62-74% protein on dry basis (Wu et al 1997). However, many extraction procedures using CGM have drawbacks, e.g., high solvent use, extraction of only α -zein due to steeping, and higher energy usage (Carter and Reck 1970). Zein can also be extracted from DDGS or dried distillers' grains (DDG). These are co-products of the dry-grind ethanol process. DDGS has the addition of condensed wet stillage and DDG does not contain condensed wet stillage (Kwiatkowski et al 2006).

The typical protein content of DDGS is in the range of 28-30%, which is higher than in DMC (Singh et al 2002). However, yields of zein from DDGS have been lower compared

to that from CGM. One of the first extractions of protein from DDGS by Wu et al (1981) found that the zein proteins in DDGS had poor solubility in aqueous alcohols, which was attributed to protein denaturation either during distillation of dry-grind ethanol after fermentation or subsequent drying of the solids. More recently, crude zein was extracted from a co-product of the whiskey production, corn distillers' grains with solubles (CDGS), which is similar to the DDGS of the dry-grind ethanol process. Yields of 3.2 to 6.6% were reported but the extracted zein contained only 37 to 57% protein (Wolf and Lawton 1997). Xu et al (2007) extracted zein from defatted DDGS using 70% ethanol and 0.25% sodium sulfite at acidic pH and obtained a solid product that was 90% protein and extracted about 44% of the protein. An extraction that utilized acetic acid as the solvent and DDG as the substrate could also extract zein protein (Selling and Woods 2008). Their protein yield was about 12%, but the protein content of the extract was low at 20%.

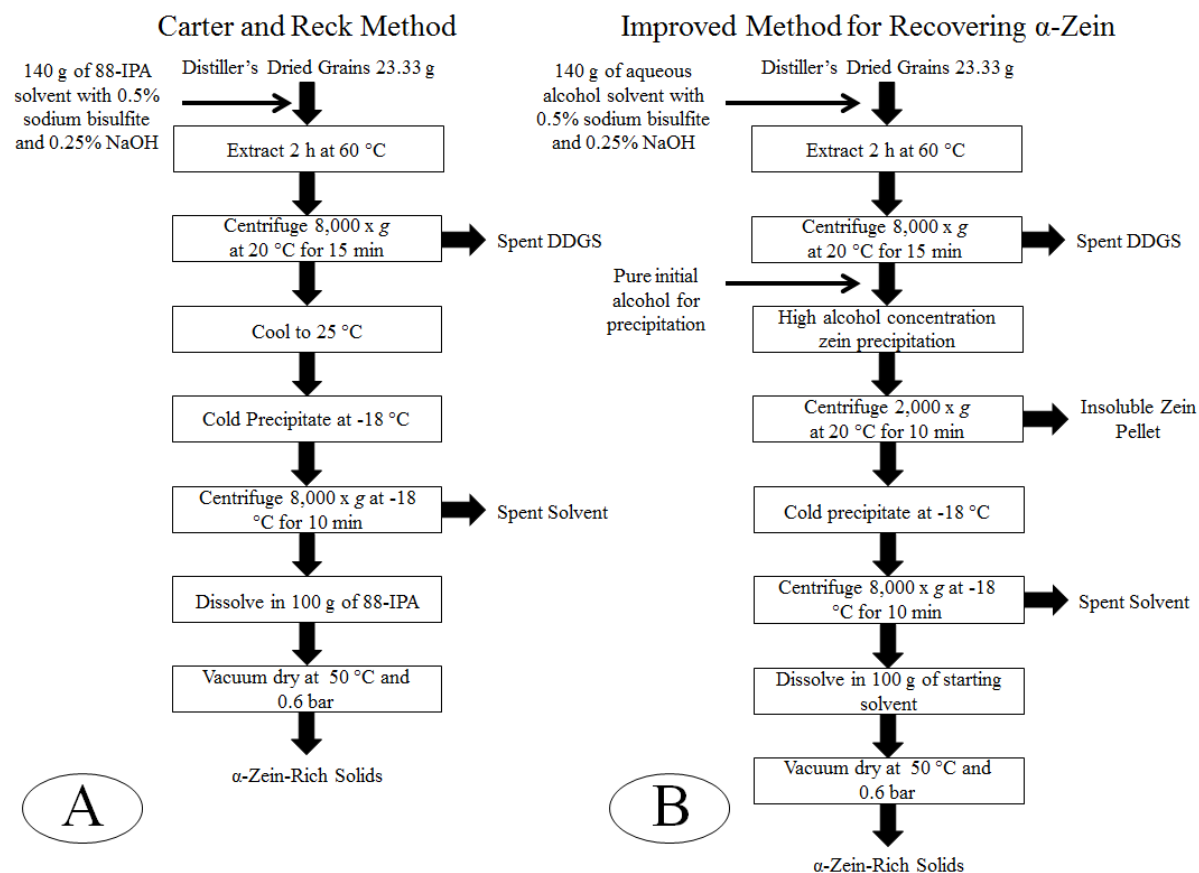
Batterman-Azcona and Hamaker (1998) have shown that cooking ground corn at temperatures as low as 100 °C can decrease the extractability of zein from protein bodies. The dry-grind corn ethanol process, of which DDGS is a co-product, involves cooking at 90-105 °C for extended lengths of time (Whitlock 2009, Robertson et al 2006). The cooking may contribute to a decrease in the zein extractability. During corn-based bioethanol fermentation, the use of proteases to release free nitrogen for yeast may also be detrimental to zein protein integrity and quality (Bothast and Schlicher 2005). High-temperature drying condition of DDGS could also be a source of damage to the zein. The solubles fraction of DDGS are low MW compounds which may hamper extraction (Kim et al 2008).

Currently, two companies in the United States have produced zein from co-product of the dry-grind ethanol process. The COPE zein extraction method, used by Prairie Gold Inc. (Bloomington, IL), obtains zein from a front-end extraction co-product (Cheryan 2009). Not only zein, but also a high value corn oil is recovered during the process (Harris et al 1947, 1949, Beckel et al 1948, Rao et al 1955, Rao and Arnold 1956ab, Johnson and Lusas 1983). The zein and corn oil mixture with high xanthophyll content were extracted simultaneously and separated by membrane technology (Cheryan 2002). The benefit of this method was to produce zein, which had not been unaltered during bioethanol process and to obtain high value corn oil. The COPE process can also be modified to produce zein products with just α -zein or a combination of α -, β -, and γ -zein.

The other new extraction procedure based on the dry-grind ethanol process was developed by POET Inc. (Sioux Falls, SD) using their no-cook BFRACTM dry-mill ethanol process to produce Dakota Gold® HPTM DDG, from which zein INVIZTM is extracted (POET 2010). This zein has been back-end extracted unlike Prairie Gold Inc.'s procedure. The zein has instead undergone ethanol fermentation process prior to being obtained in DDGS. Benefits of this method are that extraction substrate contains higher concentration of protein ($\approx 40\%$ protein) and allows for more efficient extraction than dry-milled corn. The disadvantage is that to optimize extraction efficiency, a low alcohol concentration solvent must be used and gives only one product that contains α -, β -, and γ -zein. Both products are new and experimental products are not available to have their properties evaluated.

Recently, we optimized a zein-extraction method from CGM (Fig. 4-1) by modifying the procedure of Carter and Reck (1970). The best solvent systems used were 70% (w/w)

Figure 4-1



Method A is Carter and Reck extraction method. Method B is improved method optimized by Anderson and Lamsal (in review).

aqueous 2-propanol (70-IPA) and 70% (v/v) aqueous ethanol (70-EtOH). Both solvents yielded 45% protein recovery, which was significantly higher than the 28% achieved by a commercial extraction method using 88% (w/w) aqueous 2-propanol (88-IPA). The purpose of this research was to use the optimized extraction method to increase the yield of zein and produce functional zein from DDGS. The objectives of the present study were to 1) determine the efficacy of the modified zein extraction method and solvents of Anderson and Lamsal (in review) to extract zein from DDGS; 2) evaluate the effect of DDGS pretreatment

(grinding, and or hydrolytic enzyme) on zein extraction; and 3) characterize the molecular distribution and film properties of zein extracted from DDGS.

4.4 Materials and Methods

4.4.1 Materials

DDGS was obtained from Lincolnway Energy (Nevada, IA). The CGM was obtained from Cargill Inc. (Eddyville, IA). Kobayashi zein DP was purchased from Kobayashi Perfumery Co. (Tokyo, Japan).

4.4.2 Composition Analysis

The moisture content was determined by drying the samples in a convection oven at 130 °C for 3 h (Method 44-19, AACC 2000). Crude fat contents were extracted by using hexane as solvent (Method 30-25, AACC 2000) with the Goldfish apparatus (Labconco Corp., Kansas City, MO). Mass of the dry solid samples were determined by drying overnight in an oven at 103 °C (Dickey et al 1997). All analyses were completed in duplicate and values were given on a moisture-free basis. The crude protein content of the DDGS and the extracted zein solids were determined by the Dumas nitrogen combustion method (Method 992.23, AOAC 1998) and an Elementar Vario MAX CN analyzer (Elementar Analysesysteme GmbH, Hanau, Germany). The particle sizes of the ground and unground DDGS were measured by using standard Taylor series sieves with mesh sizes of 12, 20, 30, 50, 100, 200, and pan in a RO-TAP Testing Seive Shaker.

4.4.3 Extraction of α -Zein-Rich Solids

The zein was extracted from DDGS by using two different extraction procedures as summarized in Figure 4-1. Method A used 88-IPA with 0.5% sodium bisulfite and 0.25% NaOH, and Method B used either of two solvents, 70-IPA or 70-EtOH with 0.5% sodium bisulfite and 0.25% NaOH. In Method A, zein fractions were extracted and cold precipitated; the key difference in Method B was that the solvent concentrations were increased to either 95% (v/v) aqueous ethanol or 88 (w/w) aqueous 2-propanol to precipitate β - and γ -zein leaving α -zein in solution, which was then cold precipitated. Both procedures were evaluated for DDGS with varying particle sizes and enzyme treatments.

The isolated α -zein-rich solids were described based upon protein purity, zein yield, protein recovery, and α -zein extraction efficiency. The protein purity was the percentage protein content of the isolated α -zein-rich solids. Zein yield was the percentage of α -zein-rich solids mass based on the total mass of the DDGS (db). *Zein yield, %* = [(mass α -zein-rich solids)/(total mass of DDGS(db)) \times 100%]. The protein recovery is a more accurate measurement of zein yield accounting for the percentage of protein in the α -zein-rich solids to the protein in the original DDGS (db). *Protein recovery, %* = [((protein purity, %) \times (mass α -zein-rich solids))/(total protein in DDGS mass)]. α -Zein extraction efficiency was the zein yield corrected for protein, and was a percentage of the optimal α -zein extraction value described later. *α -Zein extraction efficiency* = [((protein purity, %) \times (mass α -zein-rich solids))/(total mass of extractable α -zein protein in DDGS)].

4.4.4 Enzyme-Assisted Extraction of α -Zein

DDGS was pretreated with a mixture of enzymes cellulase and pectinase (0.4% Multifect CX GC (3200-4110 IU/g) and 0.1% Multifect Pectinase FE (145-180 U/g), both from Genencor (Rochester, NY), prior to extraction of α -zein by Methods A and B. A 0.5% (v/v) enzyme mixture was introduced to a 0.1 M sodium acetate buffer at pH 4.0 at 1:4 DDGS:solution. The enzyme and DDGS slurry was incubated with stirring at 50 °C for 2 h. The slurry was centrifuged at 8,000 x g for 15 min. After centrifuging, the solids were washed three times with 250 mL of distilled water to remove hydrolyzed sugars. Zein was then extracted following Methods A or B.

4.4.5 Total Zein in DDGS

The total zein protein in DDGS was determined based on the method of Wu et al (1997) and scaled up for ease of extraction. First, 10 g (db) of DDGS was extracted with 250 mL of 0.5 M NaCl to extract the saline-soluble proteins. The solution was stirred for 20 min at room temperature in a sealed 400-mL centrifuge tube and centrifuged at 8,000 x g for 15 min, the supernatant was collected and then the saline extraction was repeated. Total zein extraction of the remaining pellet was done with 250 mL of 55% (v/v) aqueous 2-propanol and 5% (v/v) 2-mercaptoethanol with 0.5% (w/v) sodium acetate solvent (PMA). The contents were stirred for 2 h at room temperature and then centrifuged at 8,000 x g for 15 min and the supernatant collected. The pellet was washed twice with 50 mL of PMA and the fractions were added to the first total zein collection. The total zein and residual pellet were dried and the protein contents were determined. The protein in saline-soluble protein fraction was determined by difference.

4.4.6 SDS-PAGE and Densitometry

The extracted zein samples using Methods A and B were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry following the method described by Anderson and Lamsal (in review). The total zein extract sample and the DDGS extract samples were run on different gels.

4.4.7 Zein Film Preparation

α -Zein films were made from the following α -zein-rich solid samples: (i) α -zein-rich solids extracted from DDGS with 70-EtOH with and without enzyme treatment, (ii) commercial Kobayashi zein DP, (iii) zein extracted from CGM with 70-EtOH, and (iv) α -zein-rich solids extracted from CGM with 88-IPA. The films were prepared from 0.5 g of α -zein-rich solids dissolved in 10 mL of 90% (v/v) aqueous ethanol, heated to 70 °C for 10 min, cast in Petri dishes, and dried in a vacuum oven for 1 h at 50 °C (Parris and Dickey 2001).

4.4.8 Scanning Electron Microscopy (SEM) of Zein Films

The possible variations in surface microstructure of the zein films were qualitatively evaluated by using SEM scans. A 12-mm circular portion of the film was mounted onto an aluminum stub and sputter-coated with gold/palladium (60/40) by using a Denton Desk II Sputter coater (Denton Vacuum Inc., LLC, Moorestown, NJ). Images were collected by using a JEOL 5800LV scanning electron microscope (Japan Electron Optics Laboratory, Peabody, MA) at 15kV with an OSIS ADDA II software for digital image capture (Olympus Soft Imaging Systems, ResAlta, Golden, CO.).

4.4.9 Water Vapor Permeability

Zein films were produced from α -zein-rich solids extracted from enzyme-treated DDGS with 70-EtOH, CGM extracted with 70-EtOH, and Kobayashi zein DP. These three films were chosen because they represented DDGS, CGM, and commercial zein and had uniform film thickness across the cross-section tested. The water vapor permeability calculations and procedure closely followed Yoshino et al (2002). The effective film diameter was 62 mm through which vapor diffusion took place, and average film thickness was about 75 μ m. The films were placed on top of a flat-lipped glass beaker containing 15 g of anhydrous Drierite (anhydrous calcium sulfate) and placed within a 100% humidity chamber (Fig. 4-2). The lip of beaker was vacuum-greased and the film was sealed with a custom-made flange and an O-ring to secure the film between the beaker and flange. The films were left in the chamber for 24 h. equation (Yoshino et al 2002):

$$WVP = \frac{WL}{tAP}$$

where WVP is the water vapor permeability ($10^{-9} \cdot \text{g} \cdot \text{m} / \text{m}^2 \cdot \text{s} \cdot \text{Pa}$), W is the amount of water gained by the desiccant (g), L is the film thickness (m), t is the time measured (s), A is the film cross section area (m^2), P is the difference in pressure inside and outside the beaker (Pa).

4.4.10 Zein Film Tensile Strength

Test samples of films produced from α -zein-rich solids of CGM extracted with 88-IPA and 70-EtOH, ground DDGS with and without enzyme treatment using 70-EtOH, and

Figure 4-2

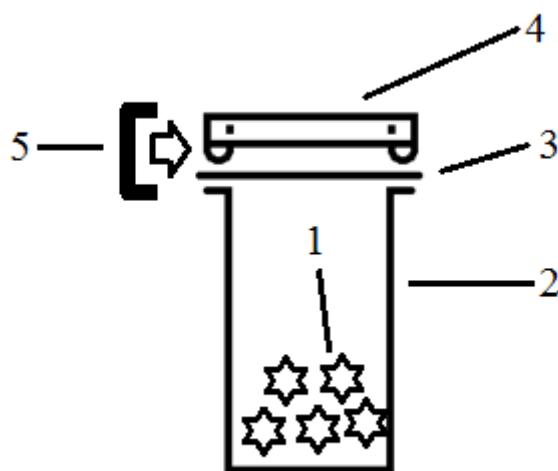


Illustration of a custom-made water vapor permeability apparatus. The depth of the beaker was 83mm and diameter 62 mm. The apparatus was placed within a 100% humidity chamber. Anhydrous calcium sulfate sealed within apparatus (1); flat-lipped beaker (2); round-cut test film (3); donut-hole flange with O-ring lip (4); and clip to sandwich film with vacuum grease between flange and flat-lipped beaker (5).

Kobayashi zein were cut using a stencil from a die conforming to ASTM D-638-V Standard (ASTM 1994). For each test film, five specimens were tested. The tested cross section was measured using a digital micrometer (Fisherbrand Traceable Digital Calipers) for width and thickness using five measurements. The tensile strength testing was carried out with an Instron Universal Testing Machine model 5569 (Instron, Canton, MA) using a gauge length of 25 mm and an extension rate of 1 mm/min. Tensile strength, Young's modulus, and percent elongation to break were recorded.

4.4.11 Statistical Analysis

The experiment used DDGS ground to two different particle sizes; each DDGS particle size was treated with or without an enzyme combination. Each DDGS substrate was

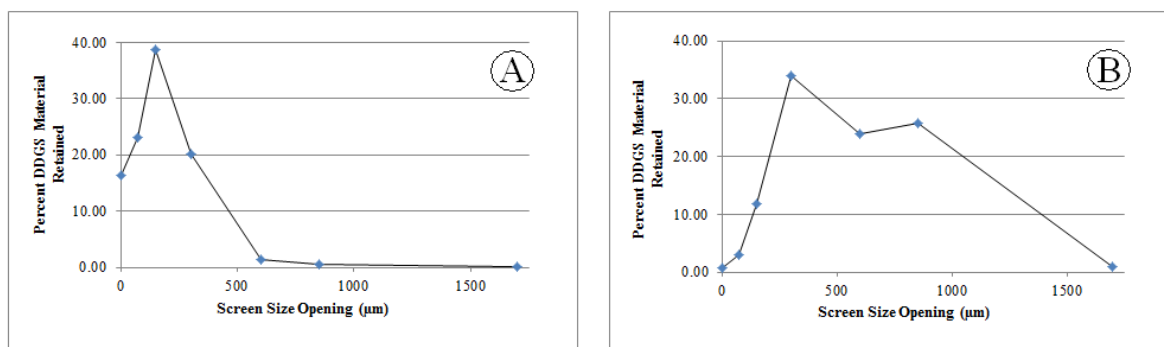
extracted with Method A by using 88-IPA or Method B by using 70-IPA or 70-EtOH. The experiment was performed with the 12 different extraction conditions following a randomized complete block design with two replications. Data were analyzed by using ANOVA with JMP v. 8.0.1 statistical software (SAS Institute, Inc., 2010). Least significant differences were determined by using the Tukey-Kramer HSD test at 5% confidence. Analytical tests/assays were carried out in triplicate or as noted.

4.5 Results and Discussion

4.5.1 Extraction of α -Zein-Rich Solids from DDGS

The DDGS used for the extraction contained 8% moisture, 29% protein (db), and 9% hexane extractable crude lipid (db). The mean particle size d_{50} (mass average) values were 1.38 mm (ground) and 2.82 mm (unground) (Fig 4-3).

Figure 4-3



Particle size distributions of ground (A) and unground DDGS (B) samples.

Protein purity, zein yield, protein recovery, and α -zein efficiency were determined for the experimental conditions and shown in Table 4-1 (see APPENDIX for ANOVA table). 88-IPA had significantly lower extraction parameter values than the other solvents. There were

Table 4-1

Yield, recovery, and protein contents of α -zein isolated from DDGS with solvent, enzyme, and particle size treatments^a

Pre-treatment	Solvent	Protein Purity, %	Zein Yield, %	Protein Recovery, %	α -Zein Efficiency, %
E-Ground	88-IPA	82.3 ABC	1.2 B	3.5 B	17.7 B
	70-IPA	84.9 AB	4.7 A	14.2 A	72.7 A
	70-EtOH	88.9 A	4.3 A	13.8 A	70.5 A
E-Unground	88-IPA	74.6 C	0.8 B	1.9 B	10.3 B
	70-IPA	85.3 AB	4.6 A	13.5 A	72.3 A
	70-EtOH	87.8 A	4.5 A	13.4 A	71.5 A
NE-Ground	88-IPA	61.4 D	1.1 B	2.5 B	12.9 B
	70-IPA	78.0 BC	5.1 A	14.2 A	72.6 A
	70-EtOH	81.7 ABC	4.8 A	14.1 A	71.9 A
NE-Unground	88-IPA	58.6 D	1.0 B	2.0 B	10.8 B
	70-IPA	78.6 BC	4.9 A	13.1 A	69.8 A
	70-EtOH	78.5 BC	4.7 A	12.6 A	67.5 A

^a Values Followed by different letters within the same column indicate significant differences ($p < 0.05$).

no significant differences between 70-IPA and 70-EtOH. The enzyme treatment did significantly increase the protein purity for 88-IPA and also increased the protein purities of both 70-IPA and 70-EtOH. This increase in protein purities was probably due to washing the DDGS substrate with water after enzyme hydrolysis cleaning it of other soluble impurities. The enzyme treatment had no significant effect on zein yield, protein recovery, and α -zein efficiency. There was also no significant effect across enzyme treatment and particle size.

The increased surface area due to grinding of the DDGS for protein purity had no significant effect across the solvents and enzyme treatment effects. For zein yield, protein recovery, and α -zein efficiency there was no significant increases due to grinding.

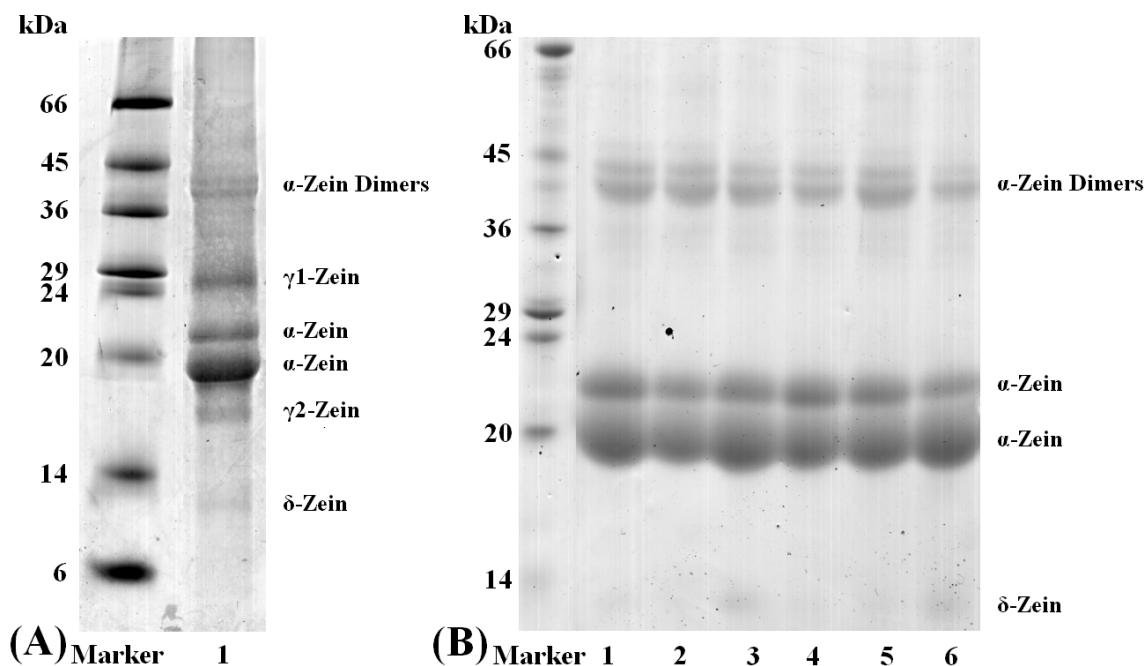
4.5.2 Total Zein Extraction from DDGS

From the total protein extraction method of Wu et al (1997), protein contents for total zeins, residual solids, and the saline-soluble fractions of DDGS were found to be 7, 18, and 4%, respectively. Over one-half of the protein within the DDGS matrix became insoluble during the dry-grind ethanol process. This insolubility of corn proteins was observed by Cookman and Glatz (2009), who found that much of the protein was insoluble unless a reducing agent was used similarly to the present study.

4.5.3 SDS-PAGE and Densitometry

The fractional analysis of the total protein described above showed that all extractable zeins in DDGS accounted for 7% of the total mass of DDGS (db). Comparing this number to the zein yields and accounting for protein purity, the highest percentage of protein recovered was from 70-IPA achieving a 4% yield of the total mass of DDGS (db) (Table 4-1). The 7% value for the total zein was higher due to the inclusion of proteins other than α -zein, (e.g., γ_1 -zein and γ_2 -zein) and potentially co-precipitated with α -zein. This speculation was supported by SDS-PAGE gels for total zein in comparison to the α -zein-rich solids (Fig. 4-4). The α -zein-rich solids contained bands at MW of 10,000, 19,000, 22,000, and 45,000 Da. Based on the nomenclature of Esen (1987, 1990) the band at MW 10,000 Da corresponded to δ -zein, the 19,000 and 22,000 Da bands accounted for α -zein, which made up the bulk of the fraction, and the MW 45,000 Da bands were dimers of the α -zein. In the gel for total zein,

Figure 4-4



SDS-PAGE protein profile of total zein and α -zein fractions isolated from DDGS. Gel A: Total zein extraction from DDGS (Lane 1). Gel B: 88-IPA NE-ground DDGS treatment (Lane 1); 70-IPA NE-ground DDGS treatment (Lane 2); 70-EtOH NE-ground DDGS treatment (Lane 3); 88-IPA E-ground DDGS treatment (Lane 4); 70-IPA E-ground DDGS treatment (Lane 5); 70-EtOH E-ground DDGS treatment (Lane 6).

it was apparent that extra bands existed at MW of 28,000 and 17,000 Da were γ_1 - and γ_2 -zein, respectively. The gel for total zein was scanned and analyzed using densitometry to determine the relative amounts of each of the zein proteins fractions. The α -zein, γ_1 -, γ_2 -, δ -, and the high-molecular-weight zein dimers made up 64, 13, 7, 5, and 11% of the total zein fraction, respectively. When determining the zein fraction homology across the total zein gel and α -zein-rich solids' gel, the α -zein-rich solids contained approximately 80% of the total zein' protein fractions. This meant that the optimal α -zein extraction yield was 5.5% protein of DDGS mass (db). This theoretical value was used to calculate the α -zein extraction efficiency as percentage of the extracted samples.

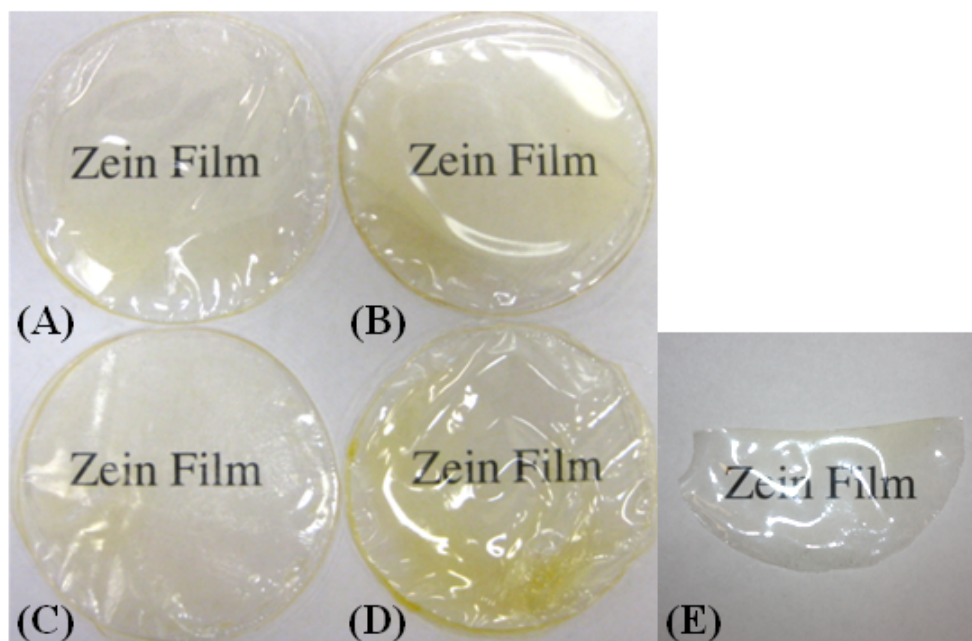
4.5.4 Zein Film Characterization

Films were produced from α -zein-rich solids extracted from DDGS using the improved method (Method B) and compared to films using commercial zein and using α -zein extracted from CGM in our lab following Method A. All α -zein-rich solids were readily soluble in 90% (v/v) ethanol and produced films on casting. The cast films are shown in Fig. 4-5. The CGM, Kobayashi, and E-ground DDGS zeins all produced very good film: they dissolved well, were highly transparent, contained small amounts of pigment, and produced mostly even film surfaces. It was not uncommon for CGM, and Kobayashi zein to produce parallel ridges due to uneven shrinking of the film at the air/zein interface. The zein from NE-ground DDGS was able to produce films, but they tended to be translucent and had uneven surfaces with irregular and random ridges (farned). This farned appearance was most likely due to residual solute impurities and because DDGS undergoes harsh conditions during the dry-grind ethanol process.

4.5.5 SEM of Zein Film Surface

The films produced from α -zein-rich solids were scanned by using SEM (bar scale 5 μm) (Fig. 4-6) to qualitatively compare differences between surfaces of films using zein extracted from DDGS and CGM. Upon the inspection of the SEM scans, irregularities or micro fractures on their surface were seen for film made from DDGS zein (Fig. 4-6). For both Films A (zein extracted from CGM with 70-EtOH) and B (zein from Kobayashi Zein DP), which were produced from zein extracted from CGM, the films were very smooth.

Figure 4-5



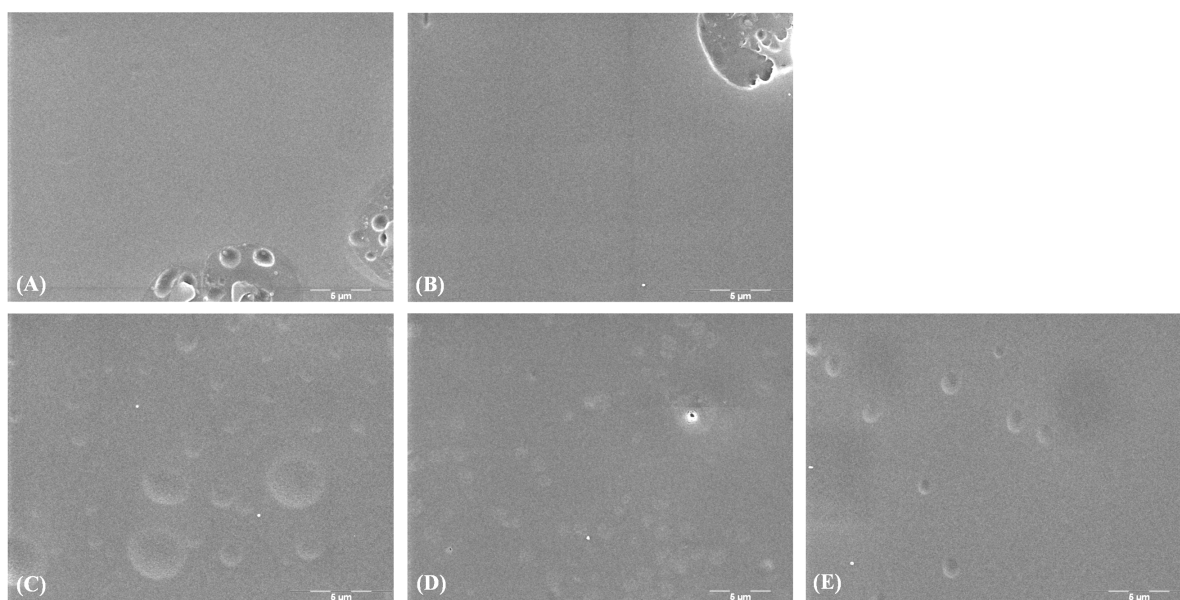
Zein films prepared from α -zein rich extracts and commercial zein. Film A, zein extracted from CGM with 70-EtOH with 0.5% sodium bisulfite and 0.25% NaOH; Film B, made from commercial Kobayashi Zein DP; Film C, zein extracted from NE-ground treatment DDGS with 70-EtOH; Film D, zein extracted from CGM using 88-IPA with 0.5% sodium bisulfite and 0.25% NaOH; Film E, zein extracted from E-ground DDGS treatment with 70-EtOH.

There were large circular irregularities with small bubbles within them, probably formed by evolution of the solvent into the gas phase when drying the films. For Films C (zein extracted from NE-ground treatment DDGS with 70-EtOH), D (zein extracted from CGM using 88-IPA), and E (zein extracted from E-ground DDGS treatment with 70-EtOH), these irregularities manifested less prominently as small circular spots scattered on the surface of the films.

4.5.6 Zein Film Water Vapor Permeability

The amounts of water vapor transmitted through the films produced from α -zein-rich solids of E-ground DDGS extracted with 70-EtOH, CGM extracted with 70-EtOH, and

Figure 4-6



SEM of zein films prepared from α -zein rich extracts and commercial zein. Film A, zein extracted from CGM with 70-EtOH with 0.5% sodium bisulfite and 0.25% NaOH; Film B, made from commercial Kobayashi Zein DP; Film C, zein extracted from NE-ground treatment DDGS with 70-EtOH; Film D, zein extracted from CGM with 88-IPA with 0.5% sodium bisulfite and 0.25% NaOH; Film E, zein extracted from E-ground DDGS treatment with 70-EtOH.

Kobayashi zein DP are presented in Table 4-2. There were no significant differences ($p < 0.05$) in water vapor transmission through the film surfaces; even DDGS films had similar water permeability properties compared to CGM films. This is important in that harsh dry-grind ethanol process to produce DDGS had not degraded α -zein protein so as to affect film water vapor permeability.

4.5.7 Zein Film Tensile Strength

The film tensile strengths, Young's moduli, and elongations to break are presented in Table 4-3. The tensile strengths of the films prepared from CGM and Kobayashi DP α -zein

Table 4-2

Water Vapor Permeability of Zein Films Prepared from DDGS, CGM, and Commercial Zein

Zein Films	Water Vapor Permeability, $\times 10^{-9} \cdot \text{g} \cdot \text{m} / \text{m}^2 \cdot \text{s} \cdot \text{Pa}$
Kobayashi Zein DP	0.042 A
CGM	0.039 A
DDGS	0.038 A

^a Values Followed by different letters within the same column indicate significant differences ($p < 0.05$).

were significantly higher ($p < 0.05$) than that of NE-ground DDGS treatment, but not different from the E-ground DDGS treatment. DDGS α -zein produced film had lower tensile strengths than the CGM and Kobayashi DP. It is unknown why the DDGS α -zein films would have lower tensile strength when they retained appreciable film-forming properties and similar to water vapor permeability values.

The Young's moduli or the flexibilities of the CGM, Kobayashi DP, and E-ground DDGS treatment film samples were significantly higher than for the NE-ground DDGS treatment. In zein isolated from NE-ground DDGS, the soluble impurities that were left in the α -zein-rich solids may have decreased film flexibility. These impurities could inhibit the formation of uniform and well-defined polymers of zein and thus create a weaker polymer matrix.

The elongations-to-break or stretchabilities of the Kobayashi DP and E-ground DDGS treatments were significantly higher than CGM and NE-ground DDGS treatment. The

Table 4-3

Tensile Strength Analysis of Films from DDGS, CGM, and Commercial Zein

Zein Films	Tensile Strength, mPa	Young's Modulus, mPa	Elongation to Break, %^b
Kobayashi DP	28.7 A	2520 A	2.70 A
CGM Zein	28.6 A	2058 A	1.43 B
DDGS with Enzyme	22.9 AB	2147 A	2.75 A
DDGS No Enzyme	19.1 B	1198 B	1.18 B

^a Values Followed by different letters within the same column indicate significant differences ($p < 0.05$).

lower elongation-to-break for the zein film made from the NE-ground DDGS treatment was difficult to explain.

4.6 Conclusions

The lab-improvised Method B using solvents 70-IPA and 70-EtOH extracted zein from DDGS as corroborated by SDS-PAGE of extracted proteins indicating α -zein bands and other sub units. From a given mass of DDGS, Method B extracted zein in quantities that were significantly higher than extracted using Method A (Table 4-1). The use of cellulase and pectinase treatment with DDGS significantly increased the protein purity. But the enzyme treatment did not improve zein yield, protein recovery, and α -zein efficiency, which indicated that intact cell wall polysaccharide components did not inhibit zein extractability. Grinding of the DDGS did not have a significant effect on protein purity, zein yield, protein recovery, or α -zein efficiency. Films from α -zein-rich solids extracted from DDGS were

successfully made; α -zein films from 70-EtOH NE-ground DDGS treatment had an irregular ferned appearance. The films produced from the same solvent extract, but with enzyme treatment appeared smooth, clear, and glossy, similar to CGM films. The SEM images of the films showed that the films were all smooth and were nearly indistinguishable from CGM films. Even though the DDGS α -zein-rich solids could produce films, the CGM films had higher tensile strengths. The water vapor permeabilities of DDGS, CGM, and commercial zein films did not differ. Overall α -zein-rich solids from E-ground DDGS treated zein samples showed properties similar to CGM and commercial zein due to their ability to produce films, and showed only slightly inferior physical characteristics.

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APPENDIX

Protein Purity, % ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	2017.5561	183.4140	48.7004
Error	12	45.1940	3.7660	Prob > F
C. Total	23	2063.7502		<.0001

Protein Purity, % Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Solvent	2	2	1032.8081	137.1165	<0.0001
Enzyme	1	1	743.3727	197.3816	<0.0001
Solvent·Enzyme	2	2	162.1784	21.5309	0.0001
Particle	1	1	30.8040	8.1791	0.0144
Solvent·Particle	2	2	34.2486	4.5469	0.0339
Enzyme·Particle	1	1	1.7013	0.4517	0.5142
Solvent·Enzyme·Particle	2	2	12.4429	1.6519	0.2324

Zein Yield, % ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	73.2144	6.6559	128.3675
Error	12	0.6222	0.05185	Prob > F
C. Total	23	74.8366		<.0001

Zein Yield, % Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Solvent	2	2	72.4249	698.4075	<0.0001
Enzyme	1	1	0.4214	8.1263	0.0146
Solvent·Enzyme	2	2	0.0731	0.7047	0.5136
Particle	1	1	0.1262	2.4330	0.1448
Solvent·Particle	2	2	0.0800	0.7712	0.4841
Enzyme·Particle	1	1	0.0028	0.0543	0.8196
Solvent·Enzyme·Particle	2	2	0.0862	0.8308	0.4593

Protein Recovery, % ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	667.0270	60.6388	95.6492
Error	12	7.6077	0.6340	Prob > F
C. Total	23	674.6347		<.0001

Protein Recovery, % Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Solvent	2	2	659.8920	520.4435	<0.0001
Enzyme	1	1	0.5470	0.8570	0.3755
Solvent·Enzyme	2	2	0.0598	0.0472	0.9541
Particle	1	1	5.3676	8.4666	0.0131
Solvent·Particle	2	2	0.0147	0.0116	0.9885
Enzyme·Particle	1	1	0.0260	0.0410	0.8429
Solvent·Enzyme·Particle	2	2	1.1299	0.8911	0.4357

α -Zein Efficiency, % ANOVA Table

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	18150.903	1650.08	91.3372
Error	12	216.790	18.07	Prob > F
C. Total	23	18367.693		<.0001

 α -Zein Efficiency, % Effect Test

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Solvent	2	2	18047.281	499.4865	<0.0001
Enzyme	1	1	15.042	0.8326	0.37950
Solvent·Enzyme	2	2	1.051	0.0291	0.97140
Particle	1	1	43.202	2.3913	0.14800
Solvent·Particle	2	2	12.521	0.3465	0.71400
Enzyme·Particle	1	1	0.960	0.0531	0.82160
Solvent·Enzyme·Particle	2	2	30.847	0.8538	0.45010

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